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TAGE KPMI
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COLLABORANT

- IN DANIA J Behel M Christiansen J Clemmensen A F Følger W Munk
Paul Møller K Sand J Ørskov
IN FINNIA P Mustakallio N Oker Blom K Setälä H Teur I Lot la
J Wallgren
IN NORVEGIA H Iker S Dick Henriksen I Kresberg D H Lahele
F Waaler C Waaler
IN SUECIA H Bergstrand B Engfeldt H Fähræus F Henschen C Hult
qu st C Kling F Linell J Mellgren S Rånström N Røgertz I Santesson
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IN VITRO INCORPORATION OF THYMIDINE- H^3 IN THE PANCREAS OF NORMAL AND OBESE-HYPERGLYCEMIC MICE

By

CLAFS HELLERSTRÖM, BO HELLMAN, SVEN BRODIN and STIG LARSSON

Received 29 iv 61

In previous autoradiographic studies the cell turnover rate in the pancreatic cells was studied by means of tritiated thymidine (Hellman, Hellerstrom & Petersson 1961). In rats, which were injected intraperitoneally on the 1st and 3rd days of life with thymidine H^3 and then killed at different times up to 5 months, differences were noted between the labelling frequency of the exocrine cells which were localized close to the islets and those which were found further away. The findings were interpreted as probably due to the particularly frequent division of the acinar cells in the region near to the islets. Since the initial higher labelling of these proximal exocrine cells may also be a result of inequalities in the thymidine distribution, consequent to the vascular pattern, it seems worthwhile also to follow the *in vitro* incorporation of tritiated thymidine in different exocrine regions. In the present investigation such an analysis has been combined with an attempt to determine whether there are any differences in percentage labelling between centrally and peripherally situated B cells in the islets of Langerhans from pancreatic glands incubated in a medium containing tritiated thymidine. The very thin pancreas of mice makes it possible to avoid slicing the organ before incubation (cf. Dickman & Morill 1957). Results are here presented both from normoglycemic animals and also from those with a severe diabetes.

plasia
and a

MATERIAL AND METHODS

Female mice 4-5 months old -
trait for hyperglycemia (ob-
Memorial Laboratories, Ba-
following groups: each con-

- (1) mice with manifest syndrome (AO mice) with an average body weight of 382 ± 11 g
- (2) lean litter mates (AN-mice) with an average body weight of 201 ± 0.2 g

The animals had free access to water and to food consisting of 71 cal per cent carbohydrate 12 cal per cent fat and 17 cal per cent protein

The animals were killed by hyperextension of the neck and the splenic part of the pancreas was then rapidly excised and divided into pieces weighing about 80 mg
 it (O_2 at $37^\circ C$)
 together with
 vessel At the
 end of the incubation period the pieces were rinsed repeatedly in isotope free buffer after which they were fixed in Carnoy's 4% thick paraffin sections were covered with Kodak AR 10 stripping film and exposed for 7 days at $4^\circ C$. The autoradiograms were developed for 8 minutes in Kodak D 19 B solution and then stained with hematoxylin

The frequency of labelled islet cells was determined at $500\times$ magnification in different regions of the first 25 islet section surfaces with a diameter of $100\ \mu$ or more which were found on microscopic examination. The regions within the islets were defined as follows:

- (A) peripheral corresponding to the outer third of the radius
- (B) intermediate corresponding to the middle third of the radius
- (C) central corresponding to the inner third of the radius

In addition to the 25 islets the frequencies of labelled exocrine cells were also calculated expressed per 1000 within different regions of arbitrarily selected visual fields (diameter approximately $400\ \mu$), each of which was adjacent to a measured islet and did not otherwise contain any islet tissue. The regions within the exocrine parenchyma were defined as follows:

- (A) near the islet, lying less than $100\ \mu$ from it
- (B) intermediate lying between $100\ \mu$ and $200\ \mu$ from the islet
- (C) far from the islet, lying within the remaining part of the visual field

The calculations of the frequencies of labelled cells within different regions of the endocrine and exocrine pancreatic parenchyma was facilitated by a squared graticule placed in the focal plane of the ocular.

RESULTS

The frequency of labelled islet cells in different regions of the islets of Langerhans is shown in Table 1. In both the AN- and the AO-mice the highest values were observed in the peripheral parts of the islets. In the AN-mice the frequency of labelled cells, expressed per 1000, was thus peripherally 5.6 ± 0.5 , and in the intermediate region 2.3 ± 0.5 ($t = 4.53$, $P < 0.001$). Labelled islet cells localized centrally were observed in only one of the ten AN mice investigated. The differences in the labelling frequencies between the centers of the islets and their peripheries were less pronounced in the obese hyperglycemic animals. In the latter animals labelled islet cells were thus found in not less than eight of the ten individuals studied. The mean figure for the central parts of the islets (2.3 ± 0.5) was, however, significantly lower than the corresponding value for the peripheries of the same islets (4.3 ± 0.4).

1 Supplied by Schwartz Laboratories, Mt Vernon, New York. The specific activity was $1.9\ C/mM$.

TABLE 1

Frequency of Labelled Islet Cells in Different Positions of the Islets in AN- and AO Mice Expressed as per 1000 For the Description of the Different Regions see Section on Material and Methods Mean Value \pm S.F.

Animal No	Frequency of labelled islet cells in different positions of the islets		
	Peripheral	Intermediate	Central
AN 1	55	56	-
AN 2	79	32	-
AN 3	74	43	-
AN 4	56	19	-
AN 5	59	-	-
AN 6	41	10	-
AN 7	60	15	52
AN 8	57	14	-
AN 9	59	13	-
AN 10	23	32	-
$M \pm S.F.$	56 ± 0.5	23 ± 0.5	0.5
AO 1	54	35	42
AO 2	26	08	13
AO 3	35	30	23
AO 4	20	05	38
AO 5	53	25	35
AO 6	43	17	-
AO 7	55	10	36
AO 8	53	13	24
AO 9	35	30	21
AO 10	31	08	-
$M \pm S.F.$	43 ± 0.4	17 ± 0.3	2.3 ± 0.5

($t = -3.0$, $P < 0.01$) An example of a labelled islet cell with a central position in an AO-mouse is given in Fig. 1

In Table 2 the frequency of labelled exocrine cells is shown with respect to their position in relation to the islets. For both types of mice significantly higher values were obtained close to the islets (region A) than further away from them (regions B and C). Thus for the AN-mice the value, expressed per 1000, for region A was 5.2 ± 0.6 , compared with 2.8 ± 0.6 in region B ($t = 2.87$, $P \approx 0.01$) and 2.7 ± 0.4 in region C ($t = 3.36$, $P < 0.01$). In Fig. 2 an example is given of an anterior

pancreas from the AO-mice, there were no mutual differences between the

$P < 0.001$

for region C ($t = 5.20$,

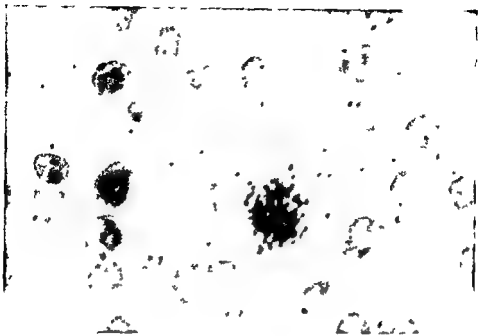


Fig 1

Labelled cell nucleus in the central part of an islet from an AO mouse ($\times 1300$)

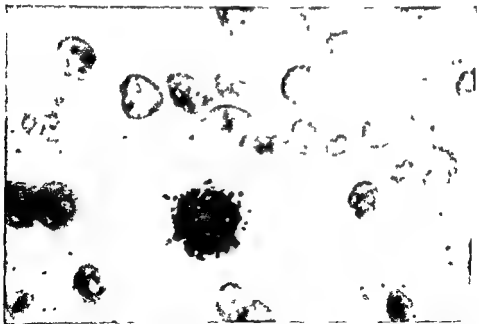


Fig 2

Strongly labelled cell nucleus in exocrine parenchyma close to an islet from an AN mouse. The islet appears in the upper part of the picture ($\times 1300$)

TABLE 2

Frequency of Labelled Exocrine Cells, Expressed as per 1000, in AN and AO Mice with Regard to their Position in Relation to the Islets For the Description of the Different Regions see Section on Material and Methods Mean Value \pm S.F.

Animal No	Frequency of labelled exocrine cells with regard to the position in relation to the islets		
	A Near the islets	B Intermediate	C Far from the islets
AN 1	78	18	29
AN 2	31	05	05
AN 3	57	44	30
AN 4	62	62	47
AN 5	26	08	12
AN 6	78	29	29
AN 7	62	16	20
AN 8	41	39	29
AN 9	47	44	51
AN 10	36	13	17
$\bar{M} \pm S.F.$	52 ± 06	28 ± 06	27 ± 04
AO 1	43	09	11
AO 2	18	09	10
AO 3	61	49	15
AO 4	31	09	14
AO 5	55	21	14
AO 6	12	06	19
AO 7	35	15	18
AO 8	49	03	10
AO 9	43	03	12
AO 10	37	06	12
$\bar{M} \pm S.F.$	40 ± 05	13 ± 04	14 ± 01

DISCUSSION

According to Hughes (1956) there are special zones for growth and decay within the islets of Langerhans. The postnatal formation of new B-cells takes place only peripherally, except in small islets, while disintegration of older cells occurs in the central parts of large islets. Statistical evidence for the existence of regional cytological differences in the islets of Langerhans has recently been presented by Hellerstrom *et al.*, 1960.

Our observations, however, do not necessarily prove the existence of a continuous centripetal migration of the islet B-cells, since there are alternative explanations for a lower cell activity in the central parts other than that these cells are the oldest (Hellerstrom, Petersson & Hellman 1960).

It is now possible to get more precise information, as to whether there exist special islet zones for cell renewal, by recording autoradiographically the incorporation of tritiated thymidine in the islet cells

(cf *Diderholm & Hellman 1960*) Even if some investigations suggest that all activity observed in the cell nuclei by autoradiographic procedures after appropriate washing and fixing techniques is not necessarily thymidine already incorporated into deoxyribonucleic acid (*Cathorn & Shooter 1960*), this technique is generally accepted as suitable for demonstrating the sites of cell renewal (cf *Hughes, Bond, Cronkite Brecher, Painter, Quastler & Sherman 1958* *Iebland, Messier & Kopriwa 1959*, *Gall & Johnson 1960*)

Since our attempts to combine the autoradiographic procedure with a selective differential cell staining of the islets have failed no precise information is available as regards the cell renewal of each islet cell type separately. It must be borne in mind that the A cells in this mouse strain represent a minority of the islet cells which in both the AN and AO mice are concentrated in the islet periphery (cf *Hellman 1961* *Hellman, Hellerstrom, Larsson & Brodin 1961*) It is hardly possible that the A cells might account for the marked regional differences in labelling frequency between the peripheral and central parts of the islets. This would suppose a very high percentage labelling of these cells in contrast to a very low or absent uptake of tritiated thymidine in the B cells. From direct mitotic counts in the islet tissue after administration of colchicine it was apparent however that the mitotic activity of the A-cells is very low in comparison with that of the B cells (*Jores & Kracht 1959*) Even if it is reasonable to accept that the differences in labelling frequency between the separate regions mainly reflect the situation in the B cells it must be realized that not only the rate of cell renewal but also the time required for DNA synthesis affect the labelling frequency of these cells. The very large discrepancy in labelling frequency between the central and peripheral parts of the islets especially in the AN mice strongly supports the view however that it is from the peripheral region that islet growth occurs. Since successive serial sections were not studied many of those islet cells which have been denoted as having an intermediate or central position are situated actually more peripherally in the islets. This means that differences in labelling frequency between the peripheral and the remaining parts of the islets are probably still more accentuated than was apparent. From the occurrence of much larger islets in the obese hyperglycemic syndrome (*Hellman, Brodin, Hellerstrom & Hellman 1961*) it follows that the chance for an islet section with a diameter $> 100 \mu$ being only a more peripheral part of an islet is greater in AO than in AN mice. Apart from the finding that islet growth probably occurs in the peripheral parts of the islets in both types of mice the figures here presented do not allow therefore any more detailed comparison between the distribution of labelled cells in the AN and AO mice.

After intraperitoneal injections of tritiated thymidine in the rat characteristic differences were noted between the labelling frequency of the exocrine cells which were localized close to the islets and those

which were found further away (Hellman, Hellerström & Petersson 1961). Near the islets the percentage number of labelled acinar cells, which was initially proportionately high, decreased more rapidly than for the cells further from the islets. Thus, the curves, expressing the values as a function of the time that had elapsed after thymidine injection intersect, the curves for the cells further from the islets being less steep. The more rapid fall in the curve, for the exocrine cells close to the islets, was interpreted as probably due to the fact that these cells divide more frequently. The results of the present *in vitro* experiment strongly support such a view. In studying the incorporation of tritiated thymidine *in vitro*, it could be excluded that the initial greater labelling frequency of the acinar cells near to the islets might be due to inequalities in the thymidine distribution consequent upon the vascular pattern. As was previously pointed out regional differences in the DNA metabolism of the exocrine tissue may reflect the effect of a high insulin titer in the vicinity of the islets (Hellman, Hellerström & Petersson 1961). The insulin concentration is probably especially high around the islets of Langerhans in the obese hyperglycemic mice with their raised B cell activity (Hellman & Petersson 1960, Hellman, Hellerström, Larsson & Brodin 1961). It is therefore of interest that the proportionately greatest differences in labelling frequency near and far from the islets seem to occur in these animals.

SUMMARY

In vitro incorporation of tritiated thymidine into the pancreas was studied in normal and obese hyperglycemic mice, by means of autoradiography. The labelled islet cells were mainly localized to the periphery of the islet. In the exocrine parenchyma the labelling frequency was higher in the immediate vicinity of the islets than in more distant positions. The marked discrepancy in the labelling frequency between different regions of the islets supports the view that renewal of B cells takes place mainly in the peripheral parts of the islets.

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SUDDEN DEATH DUE TO STINGS FROM BEES AND WASPS

Report on Four New Cases, Three with Autopsy

By

OLAF MAHRF JENSEN

Received 7/1/61

Stings from bees and wasps, the best known members of the insect order Hymenoptera, are so common that almost everybody is familiar with their inconvenient effects through painful self experience. Several more or (especially) less useful therapeutics are advocated by lay people, and medical care is rarely needed. This might explain that pertinent information concerning hymenopter stings are rarely mentioned in medical textbooks and periodicals, in spite of the fact, that in temperate zones, including i.e. Scandinavia and The United States, stings from hymenoptera causes more deaths than for example bites of poisonous snakes.

Ordinary local reaction The symptoms following one or a few bee- or wasp stings vary appreciably from individual to individual and even from one sting to the other in the same person. Usually, immediately after the sting an intense burning local pain occurs, some minutes later followed by swelling of the part and itching. A red zone of varying extension surrounds the place of the sting, in the centre a tiny white spot is usually detectable. In the case of bee stings the sting itself is left, perforating the epidermis to a depth of 2-3 mm. The sting cannot be easily brushed off as the lancet is provided with barbes. The wasp on the contrary removes the sting, when poison is placed. The local reaction, usually limited to an area of a few square centimeters, most

... may tell a little indisposed with headache

Extraordinary Local or General Reactions In some cases more severe reactions occur. Thompson (1933) grouped these in order of increasing rarity as (1) abnormal swelling and irritation, lasting an abnormal time, (2) massive urticaria and oedema supervening, (3) shock and collapse or even loss of consciousness with hypotonia and tachycardia, followed by mild pyrexia and sometimes diarrhoea and polyuria, (4) true dyspnoea soon after sting, (5) sudden severe general symptoms

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might be sensitized by single or repeated stings or by inhalation of desquamations of bees and other insects

In studies on extracts from pulped bodies of hymenoptera *Foubert & Slier* (1958) found that these insects contained common antigens and antigens specific for each genus, and separated four to six antigenic fractions, some of them probably immunologically identical in all insects studied. They suggested use of an antigen mixture for desensitization.

Mueller (1959) found that 75 per cent of his 84 patients with systemic allergic reactions to insect stings had a family or personal history of other allergy, and advised desensibilizing treatment (see later) to all patients displaying systemic reactions to stings or hypersensitivity to intracutaneous injection of whole extract of bees and wasps. Of 76 patients so treated, 30 had subsequent stings with no or only slight systemic reactions.

A unique case was described by *Wolf* (1950) in discussing *Swinnny's* paper (1950). A young farmer was given wasp desensitizing treatment. The first re severe a sting

In Denmark specific desensitization has been given since 1947 by *Bruun* (1950). He reports on a 43 year old woman, a bee-keepers wife, who for 13 years had "hay fever" and allergy to bee stings and several times had been almost moribund after stings. Desensitizing treatment for four years had excellent results, the patient thereafter responded normally to stings.

Thus antigen reacts in the sensitive person by both immediate and delayed reaction. The antigen is species specific, sometimes order specific, some antigen fractions probably being identical in all hymenoptera. It is proteinbound, thermostable, non dialyzable. Passive transfer (Prausnitz-Kustner reaction) is possible. Desensitization in persons hypersensitive to hymenoptera stings has proved successful.

DEATH DUE TO STINGS FROM BEES AND WASPS

No reliable informations concerning mortality or even total number of fatalities due to stings of hymenoptera are at hand.

In

On the basis of death certificates 1950-1954 Hymenoptera (bees, wasps, hornets, yellow jackets, and ants) caused 86 deaths. In comparison, poisonous snakes accounted for 71 deaths. Males were fatally stung and bitten more often than females. Adults were the most frequent victims of the stings by hymenoptera, whereas children and adolescents more often subcombed to snake and

hot flush over the whole body severe dyspnoea wheezing and coughing almost choking general trembling great anxiety, sometimes followed by coma. In women this may be accompanied by uterine contractions. This severe condition may last up to three or four days. (6) death (see later)

According to *Thompson* the first three categories are seen in about 2 per cent of all stings, whereas the more severe reactions are rare. *Thompson* maintained that children are rarely stung whereas nervous and irritable people who perspire freely are more liable victims. *Thompson* found the connection with the thyroid gland obvious and interesting.

CHEMISTRY OF BEE VENOM

Investigations on the chemical nature of bee venom have been subject to great discrepancies. *Fanger* (1897) analyzing venom from 12 000 bees (*apis mellifica*) extracted a basic alkaloid as the essential substance but ascribed no toxic effect to the slight contents of formic acid.

Phisalix (1902) separated a histotoxic factor a convulsory factor and a paralyzing factor with different chemical physical properties.

Reinert (1936) is displaying haemolytic histamine $1\frac{1}{2}$ per cent in raw venom.

Ackermann & Mauer (1944) however maintained that the venom did not contain histamine but that this was liberated in the tissues by the venom.

Recent investigations by electrophoresis paper chromatography and electron microscopy reviewed by *Holmes* (1955) have demonstrated a dehydrogenase inhibitor and a hyaluronidase in the bee venom.—The venom of the common wasp (*Vespa vulgaris*) contains high concentrations of histamine and furthermore serotonin (5 hydroxytryptamine) and a substance designated kinin a slowly dialysable peptide with action on isolated guinea pig intestine. The two substances are absent in bee venom.

PATHOPHYSIOLOGICAL EFFECT AND ANTIGEN PROPERTIES OF BEE AND WASP STINGS

The response to bee venom is much similar to that of snake venom (*Marshall* 1957).—Applied intravenously bee venom induces fall in blood pressure dyspnoea and intestinal peristalsis in dogs and rabbits (*Essex et al* 1930) and prolonged coagulation time due to thrombokinase inhibition (*Dyckhoff & Marx* 1943). Horses may show serum cholinesterase inhibition after a single bee sting (*Zeller Kocher & Maritz* 1944).

The more severe reactions which may sometimes follow stings from hymenoptera and which have been ascribed to either histamine (*Aldberg & Kellaway* 1937) or serotonin (*West* 1937) (experiments in rats) might also be explained as an anaphylactic shock due to an antigen reaction in a sensitized organism.

Benson (1939) maintained that the venom was not itself a true antigen but that during stinging minute amounts of antigen from the body of the bee might be induced which could sensitise people stung. People

Wasp Stings with Autopsy Performed

Symptoms and signs	Interval (min) sting-death	Postmortem findings
Cyanotic lips cold sweat pallor dyspnoea abdominal cramps 1 uneven breathings	30	Sting verified macroscopically Congestion to brain vessels and venous sinuses cerebral ventricles contained reddish serous liquid
Collapse Pulse and respiration re occurred after treatment Patient remained unconscious	5 days	Bronchopneumonia (brain not examined) 1) Uterus removed 12 years previously for fibroids
Headache cyanosis collapse	few	Heart dilated Lymphatic tissue in throat hyperplastic
Immediately dyspnoea and cyanosis	20	Glottal oedema Visceral congestion Sting verified microscopically
Fainting froth at mouth cyanosis unconsciousness convulsions	20	Glottal oedema pulmonary congestion and oedema Petechial haemorrhages in gastro duodenal mucosa and liver capsule
(not stated)	few	Visceral congestion Hypertrophic heart with slight myofibrosis Severe non toxic goitre pulmonary emphysema Sting verified microscopically
(not stated)	few	Pulmonary oedema and emphysema splenic congestion Hypertrophic heart and slight arteriosclerosis Sting verified microscopically
Feeling sick — Later found unconscious	15	No organic lesions found
Congestion to face collapse	20	Sting verified macroscopically Pulmonary oedema and congestion Persisting thymus Excess of clear fluid in pericardial sac Coronary vessels healthy
Fainting vomitus pallor with cyanosis of lips profuse perspiration	60	Glottal oedema petechial haemorrhages in skin and epicardium Visceral congestion Pulmonary emphysema Fatty degeneration of liver Sting verified microscopically
Collapse cyanosis uneven breathings no pulse Warded in hospital	5 days	Petechial haemorrhages in brain meninges epi and endocardium pleurae Gastric mucosa and in pelvic mucosa in kidneys Pulmonary oedema and bronchopneumonia Fatty degeneration of liver
Cyanosis unconsciousness	20	Slight glottal oedema severe pulmonary oedema and emphysema
Immediately weakness vomitus abdominal cramps coldness froth at mouth gasping convulsions	30	Heart dilated Mucus in tracheobronchial tree Glottal oedema Cerebral oedema

TABLE
Survey of 20 Fatal Cases of Bee and

Year or Year	Age Sex	Previ- ous stings	Previous reaction to sting	Site of fatal sting	Bee or wasp	Interval (min.) to symptom
Delpech 1880	6 M		yes	forehead	b	few
Jansen 1921	39 F	1)	yes	left leg	b	15
Vegelin 1933	36 M				b	few
Vegelin 1933	40 M			neck		0
Halperin 1936	40 M			neck	w	5
Halperin 1936	46 M		yes	right arm	b	few
Halperin 1936	52 M		yes	face or neck	b	few
Jyke 1941	60 F			neck back	w	few
Jyke 1941	44 M	—		right temple	w	few
Vegelin 1948	33 M			left hand	w	30
Vegelin 1948	38 M		n	head	b	few
Vegelin 1948	39 F	—	yes	right arm	w	few
Chenken et al 1953	21 M		yes	right temple	b	0

1 (cont.)

Wasp Stings with Autopsy Performed

Symptoms and signs	Interval (min.) sting to death	Postmortem findings
Cyanoanosis	30	Gloital and laryngeal oedema pulmonary emphysema
Fainting pallor with cyanosis unconsciousness	15	Sting verified macroscopically Petechial haemorrhages in conjunctival sac and endocardium Slight oedema of uvula Mucus and some aspirated food in tracheobronchial tree Pulmonary congestion and emphysema Coronary atheromatosis
Comitus Intense convulsions	15	Visceral congestion cerebral and pulmonary oedema petechiae and gastric mucosa small recent infarctions in lungs and spleen
Cyanosis perspiration froth at mouth gasping	45	Visceral congestion cerebral and pulmonary oedema petechiae in brain pleurae and adrenals
Clonias trembling pallor uneven breathings	30	Discoloration and oedema of tip of 4th toe—Non toxic adenomatous goitre Pulmonary congestion Biliary calculi Sting verified microscopically Pharmacological examination (see text)
Collapse	few	Pulmonary emphysema Right side of heart hypertrophic (see text)
Froth at mouth collapse	30	Heart dilated Pulmonary oedema Subcutaneous haemorrhage on right arm (see text)

prefer to indicate a possible heart disease or other commonly acceptable cause on the death certificate. The author's personal view is, that a more reliable account on frequency of death from bee and wasp stings might be obtained from newspapers than from an analysis of death certificates.

A survey of 30 previously reported fatal cases with 4 new cases added is given in Tables 1 and 2. Before commenting on these, the author's 4 cases are here reported in details.

REPORT ON FOUR NEW CASES THREE WITH COMPLETE AUTOPSY

1 131/59

in 1959 at 10/55 am while changing to her usual wooden working shoes she uttered "Oh I was stung in my toe." A comrade also noticed the insect (bee or wasp) on the stocking on the floor. The patient sat down to examine the toe

TABLE
 Survey of 20 Fatal Cases of Bee and Wasp Stings

Author Year	Age Sex	Previous illness	Previous reaction to sting	Site of fatal sting	Bee or wasp	Interval (min.) sting, death
Rehaneck 1957	37 M		yes	temple	b	15
Marshall 1957	52 M		no	chest neck and arm	w	few
Vasilu & Bucarest 1960	22 M			right cheek	w	
Ihuna 1960	47 M		yes	left ear	b	few
Myhre Jensen 1961	51 I	—	no	4th right toe	w	few
Myhre Jensen 1961	57 M	—		temple	b	few
Myhre Jensen 1961	62 I	—	no	head(?)	b	few

spider venoms. 88 per cent of deaths from hymenopter stings occurred within one hour after the sting, on the contrary several hours elapsed between the snake and spider bites and the victims deaths—Parrish attributed deaths following insect stings to anaphylaxis resulting from insect allergy.

In Denmark bites from the viper is traditionally believed to be very dangerous whereas most people almost deny that bee and wasp stings may cause death. Yet, fatalities from stings are in fact more frequent than deaths from viper bites. This does not indicate anything concerning mortality, as bites from vipers are just as rare as stings from hymenoptera are common.

In "Causes Of Death In The Kingdom Of Denmark" from 1951-59 the total number of deaths from poisonous animals (N 978) is 6 four of which are known to be caused by stings from Hymenoptera.

In conjunction with Swinny, Schenken et al (1953), Parrish and others, the author is convinced, however, that deaths from bee and wasp stings are much more common than generally believed. Presumably some doctors hesitate to recognize a sting as the cause of death, but



Fig 1

Sting canal. A delicate defect in the epidermis with slight lymphocytic infiltration
x Giesson stain (X 64)

After a few minutes she resumed her seat. The body her face turned

A complete and painstaking medico legal autopsy was performed 26 hours post mortem with complete histologic examination.

The corpse was thin and meager (168 cm 52 kg) without lesions except for the tip of the fourth right toe which was discoloured, faintly reddish and moderately swollen in the terminal segment only. In the centre of the discoloured part, 3 mm lateral to the tip of the toe a yellow point 1 mm in size was detectable. No visible perforation of the skin and no sting was found.

The organs displayed acute congestion, notably in the lungs, but no glottal oedema or pulmonary emphysema were found. The heart (weight 250 g) and brain were normal. The right thyroid lobe was slightly enlarged, the whole gland weighed 54 g and contained a few very small cysts separated by moderately firm parenchyma. The gall bladder contained some stones. The left ovary contained a liquid content.

Microscopic

Survey of 14 Fatal Cases of Bee and Wasp Stings without Autopsy Performed

Author Year	Age Sex	Previ- ous illness	Previous reaction to sting	Site of fatal sting	Bee or wasp	Interval (min.) slung symt	Symptoms shown and further remarks
Lassen 1879	37 M		yes	cheek	b	0	Immediately perspiration pallor froth at mouth unconsciousness Death in few minutes
Kaarsberg 1904	60 M		yes	hips	b	0	Immediately unconsciousness Death within 5 min
Arentsen et al 1923	35 F	—	yes		w	few	Vomitus cyanosis collapse unconsciousness Death within 60 minutes
Arentsen et al 1923	32 F				w		Froth at mouth involuntary urination and defecation Death within 30 minutes
Kalperin 1936	8 F			right 2nd finger	b	few	Dyspnoea and cyanosis unconsciousness Death within 30 minutes
Alexander 1938	56 F	1)		1st toe	w	20	Collapse unconsciousness Death within 60 minutes
Hallas 1943	45 F	—	yes	arm	w	few	Oppression in chest dyspnoea cyanosis froth at mouth Death within 20 minutes
Hallas 1943	50 M	—	yes	ear	w	0	Immediately collapse Death within 15 minutes
Hallas 1943	46 M	2)		chin	b	30	Dyspnoea cyanosis collapse, no pulse or respiration Death within 60 minutes
Hallas 1943	41 F	3)	no sting	leg	b	few	Cyanosis collapse unconsciousness Death within 20 minutes
Swinnys 1950	42 M	—	yes	neck forehead and axilla			Cyanosis involuntary urination frothy blood at mouth unconsciousness Death within 5 minutes
Jex Blake 1942	45 M	—	no	face	w	3	Collapse Artificial respiration without effect Death within 60 minutes
Jex Blake 1942	55 M		yes	neck	b	few	Collapse Death within 15 minutes
McIure Jensen 1961	67 F	4)		fingers	w	few	Severe asthmatic attack collapse artificial respiration and oxygen without effect Death within few minutes

1) Suffering from cardiac disease and hypertension

2) Asthma no attack since 1941

3) Slight cardiac symptoms giving no serious attacks

4) Severe asthma for several years

Chronic leg ulcer

Toxicologic Examination In order to ascertain a possible inhibition of choline sterase activity samples of blood were taken from right (1) and left (2) femoral vein and from a normal control (3) Furthermore tissue from the tip of the affected toe was taken weighed (655 mg) crushed and mixed with 6 5 ml of the control blood (4) Tissue from the contralateral toe was treated identically (710 mg tissue to 7 1 ml control blood) (5)

The five samples were kindly examined by the Pharmacological Department University of Copenhagen (Method Jensen Holm Lausen Mithers and Møller 1959)

The results displayed only minor differences within normal limits but the lowest activity of acetylcholinesterase was found in the sample (4), which might suggest an acetylcholinesterase inhibiting factor in the sting

Analysis of histamine in the samples proved unsuccessful because of shortage of blood available for analysis

The microscopic examination of the sting revealed a sting canal) the insurance company, and liability according to the workmen's compensation act was granted

Epicrisis 51-year old healthy woman, previously stung without alarming reactions, now stung by a wasp followed by rapid cardio-pulmonary shock and dead within 35 minutes — Autopsy revealed acute congestion of lungs, a nodular goitre, but no glottal or pulmonary oedema or emphysema — Microscopic examination displayed the sting canal, penetrating the epidermis and a necrotic underlying sweat gland — Acetylcholinesterase activity slightly lower in tissue from the site of the sting than from the contralateral control — The case was accepted as an accident of the insurance company

II 1953

A 52 year old woman

A woman who had been stung several times heard her

On the

On the
was con
compens

Epicrisis 62-year old woman, previously stung without alarming reactions Obviously stung by bees in the scalp, followed by fainting, unconsciousness, foam at the mouth and death within 30 minutes — Postmortem examination uncontributory except for moderate pulmo-

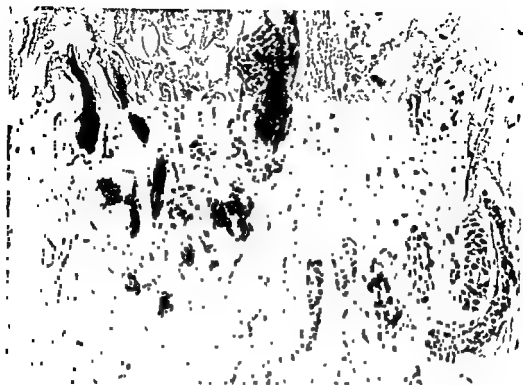


Fig. 2

Necrosis of an underlying sweat gland tube with oedema and hyperaemia in the surrounding loose connective tissue—Haematoxylin eosin stain ($\times 64$)

the necrotic gland was slightly oedematous, whereas no cellular infiltration was present. The capillaries in and close to the necrosis were dilated and engorged with erythrocytes, but no damage to capillary endothelium was apparent—Other sweat glands in the same vision field were perfectly normal. The lesions described could be followed in 4-5 consecutive sections.

Sample	Sort of sample	Enzyme activity (μ mol/min/ml blood)	
(1)	blood, right fem vein	8.69	9.0
(2)	blood left fem vein	9.30	
		8.84	9.1
		9.25	
(3)	control blood	0.00	5.9
		5.79	
(4)	tissue (sting) + control blood	5.48	5.6
		5.64	
(5)	tissue (contralateral) + control blood	6.60	6.4
		11.15	

Preparations from the left 4th toe (control) revealed normal structure. The thyroid displayed a benign non-toxic adenomatous architecture without inflammation. Complete histological examination including the central nervous system, pituitary gland, heart, liver, spleen, kidneys, adrenals, pancreas, uterus with cervix (with only slight, superficial erosion) was uncontributory. Both lungs showed severe acute congestion. The left ovary contained some minute follicle cysts.

Toxicologic Examination In order to ascertain a possible inhibition of cholinesterase activity samples of blood were taken from right (1) and left (2) femoral vein and from a normal control (3). Furthermore tissue from the tip of the affected toe was taken weighed (655 mg), crushed and mixed with 0.5 ml of the control blood (4). Tissue from the contralateral toe was treated identically (710 mg tissue to 7.1 ml control blood) (5).

The five samples were kindly examined by the Pharmacological Department University of Copenhagen (Method Jensen Holm Lausen Milthers and Møller 1949).

The results displayed only minor differences within normal limits but the lowest activity of acetylcholinesterase was found in the sample (4), which might suggest an acetylcholinesterase inhibiting factor in the sting.

Analysis of histamine in the samples proved unsuccessful because of shortage of blood available for analysis.

The main conclusion based on the detailed case history, the gross and microscopic autopsy findings and the important fact that no other disease was revealed was that the death was caused by a sting of wasp (No sting found in the sting canal). The death was thus classified as an accident this was accepted by the insurance company, and liability according to the workmen's compensation act was granted.

Epitaph 51 year old healthy woman, previously stung without alarming reactions, now stung by a wasp followed by rapid cardio-pulmonary shock and dead within 35 minutes — Autopsy revealed acute congestion of lungs, a nodular goitre, but no glottal or pulmonary oedema or emphysema — Microscopic examination displayed the sting canal, penetrating the epidermis and a necrotic underlying sweat gland — Acetylcholinesterase activity slightly lower in tissue from the site of the sting than from the contralateral control — The case was accepted as an accident of the insurance company.

II 61/53

A 62 year old woman

stung herself several times heard her cry that she had got bees in her hair, telling minutes later she complained of feebleness & quickly carried to the house but was out. Shortly afterwards her respirations minutes later she was dead. Between 15 and 30 minutes had passed from the moment the bees got into her hair until death was ascertained.

The lungs were slightly congested and oedematous but no emphysema or glottal oedema was present. The heart was moderately dilated weight 375 g. The coronary arteries and the aorta displayed only slight atheromatosis but no calcifications or stenosis. The cerebral arteries were perfectly normal. The remaining organs were normal. No goitre was found.

On account of the rapid death was included compensation.

Epitaph 62-year old woman, previously stung without alarming reactions. Obviously stung by bees in the scalp, followed by fainting, unconsciousness from at the mouth and death within 30 minutes — Postmortem examination uncontributory except for moderate pulmo-

nary congestion and oedema—The case was accepted as an accident of the insurance company

III 10/37

57 year old previously perfectly healthy man without information concerning previous stings—On July 11th while working in the garden together with his wife he suddenly shouted that he was stung in the temple by a bee. His wife pulled out the sting but just in the same he collapsed. A doctor arrived immediately but death had already occurred—Only a few minutes had passed from sting until death

Postmortem examination (5 days after death) A thorough medico legal autopsy was performed. The state of nourishment was well above normal (179 cm 80 kg). In the left temple some minor discolouration was faintly seen but no sting was found. Putrefaction however complicated the examination.

The lungs were slightly emphysematous. The heart weighed 300 g the right ventricle was moderately hypertrophic. The coronary arteries and the aorta showed only very slight atheromatosis. The cerebral arteries none at all—The organs displayed putrefaction but nothing abnormal. No histologic examination was performed.

It was concluded that as postmortem examination had not revealed any disease which might explain the sudden death this had most likely been caused by a sting of a bee.

Epicrisis 57-year old healthy man, stung in the temple, presumably by a bee as the sting was stated to be removed. Few minutes later collapse and death. Postmortem examination uncontributory.

IV E 44/59

67 year old woman with chronic asthma-bronchitis for which she received disablement pension since 1932 successfully treated with cortisone since March 1958. Two months later she sustained a left calcaneus fracture and was warded in hospital for 2 months. Cortisone was discontinued and dyspnoea coughing and expectoration increased but was benefited from theophylline and various expectorants—Laboratory investigations showed hemoglobin 93-110 per cent sedimentation rate 18 mm/h urine normal. ECG right sided cardiac hypertrophy. X-ray of thorax some pulmonary emphysema blood pressure 130/90. The fracture healed. The pulmonary state remained unchanged.

On September 7th at 0.30 p.m. when she was standing in the kitchen she uttered a cry and told her husband that a wasp had stung her in the finger. She went for ammonia water but before achieving her purpose she had a severe attack of asthma. A doctor arrived in a few minutes and gave a stimulation injection (nicotamide?) and artificial respiration were given in the ambulance but on arrival to hospital death had occurred. Only a few minutes had passed from sting until death.

At the medico legal investigation of the body the cause of death was stated as shock and acute attack of asthma due to sting of a wasp in the finger. Autopsy was not performed.

Epicrisis 67-year old woman disabled from asthma bronchitis, stung in the finger by a wasp died few minutes later in a severe attack of asthma—No autopsy.

ANALYSIS OF CLINICAL DATA IN 34 PUBLISHED FATAL CASES

The author has found 30 cases of death from hymenoptera stings published by others (see Table 1 and 2). Including the 4 cases reported above a total of 34 cases are available for review.

Sex 22 males 12 females

Age One fatal case concerned a boy of 3 years, another a girl of 8 years. Two were men of 22 and 23 years. Nine victims were in the age group 30-39 yrs, 10 between 40 and 49 yrs, 7 between 50 and 59 yrs and 4 in the age group 60-69 yrs. This age distribution confirms the statement of Parrish (1959) that almost only adults are dying from bee and wasp stings. In his 86 cases collected from United States' death statistics 5 were younger than 10 years, one between 10 and 19 years. The highest incidence was also here in the third to fifth decades (about 69 per cent).

Species of hymenopter 15 fatalities were due to stings of wasps (*Vespa vulgaris*, hornets, yellow jackets). 17 persons were stung to death by bees (*Apis mellifica*, *Apis mellifica adansonii*). In 2 cases the species of hymenopter was not stated.

Previous illness Only 5 of 34 patients here reviewed had a history of previous or chronic illness, 2 of them had bronchial asthma.

Previous stings 16 patients had a history of previous allergic reactions to stings. In one case a previous sting had caused considerable oedema of the whole arm, in 12 cases dramatic systemic reactions had occurred.

In the second case of *Jex Blake* (1942) a 55 year old man who had previously had numerous stings without noticeable symptoms suddenly changed his way of re-

Five persons had been stung previously without any systemic reaction. One was known to have never been stung before. In 13 cases information concerning previous stings was missing.

Localisation of fatal sting In 20 cases the fatal sting (or stings) was placed in the head or neck. In 11 cases other localisations were indicated, most often on the extremities. The localisation was not stated in 3 cases.

Time relations The interval from sting to beginning of severe symptoms ranged from "immediate" to 30 minutes, most often 2 to 10 minutes. The interval from sting to death ranged from "few" to 60 minutes, except for only two patients, who remained unconscious for 5 days, eventually succumbing to complicating bronchopneumonia. In Parrish's material 88 per cent died within 1 hour after the fatal sting.

Symptoms and signs The clinical pictures were almost identical in all fatal cases with symptoms from the respiratory and circulatory systems dominating: dyspnoea, uneven respirations, froth at mouth and nose, cyanosis, tachypnoea, tachycardia, irregular pulse, tremulousness, muscular trembling or even convulsions.

nary congestion and oedema —The case was accepted as an accident of the insurance company

III 10/37

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IV 14/59

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Sex 22 males, 12 females

usually few or inconspicuous, sometimes not even mentioned. Severe local oedema, general rash or itching were not found. The symptoms in the fatal cases were those of a rapid, general shock with sudden onset, fainting and unconsciousness, sometimes twitchings, superficial uneven breathings, dyspnoea or cyanosis. The clinical picture was that of a sudden anaphylactic reaction.

A very important detail was the fact that the fatal issue was very rapid, within 60 minutes in all cases except two which were rather untypical as death here was due to complicating pneumonia.

Wegelin considered the following factors of possible predominant importance in pathogenesis of fatal cases:

1 *Localisation of sting in specially dangerous regions*. Sting in the tongue, palate and throat has always been considered dangerous, because of the consequent suffocation from obstruction of the air ways following local swellings (glottal oedema). Fatalities of this type are especially apt to occur when people get a bee or wasp in the mouth, for instance during eating sweets or bread with jam, etc. A survey of fatal cases (see Table 1 and 2), however, reveal that cases of this type are in fact not at all frequent. Only two such cases are on record (*Delpech*), both having an atypical clinical picture: death occurred after some hours from sting (one case after 27 hours).

Considering the cases mentioned here it is obvious that stings in the regions mentioned above do not account for many deaths. Only one person with sting in the lips, causing no airway obstruction, is included in the material.

Furthermore it has been suggested (*Roch* 1928) that stings in the face, head and neck are particularly dangerous, maybe on account of a direct diffusion of the venom to the mucosal membranes in the upper airway causing oedema with suffocation. In 19 of the 33 fatal cases here recorded the fatal sting was located to head or neck. In 11 cases another localisation was mentioned while the localisation was not stated in the remaining 3 cases. So there is a rather high frequency of stings in head or neck among the fatal cases. This does not indicate, however, as has been suggested, that a direct diffusion of venom to mucosal membranes of the upper airways with consequent oedema should be responsible. Glottal oedema is not seen in all cases with sting in head or neck, and was not the only cause of death in any of the cases. On the other hand sting in finger or anywhere may be accompanied by glottal oedema. In fact one should expect the head and neck to be the most likely site of all stings simply because these regions constitute more than 50 per cent of the naked area when the protective effect of the clothes is considered.

2 As a second possibility Wegelin stressed that accidental *intra cutaneous inoculation* might explain the fatal cases. This was an old concept generally accepted by lay people. This might explain that systemic reactions occur very rapid in spite of absent or moderate local symptoms.

were reported—In 7 cases the reports were quite laconic collapse with death occurring within a few minutes

Delpech (1880) reported 11 cases of death from bee sting One is included in Table 1, whereas the others are omitted as informations concerning age sex, previous illness or previous reactions to stings are missing So much however could be deduced that all 10 deaths occurred in adults in 7 cases within 1 hour after the sting while in 3 the interval was up to about one day Three persons had several stings Two were stung in the throat or palate with consequent choking One of these died after some hours the other after 27 hours in spite of tracheotomy

ANALYSIS OF AUTOPSY FINDINGS IN 20 CASES

Reports on autopsy findings were included in 17 of the previously published cases, but in some only partial necropsy was performed Including the author's 3 cases with complete autopsy, 20 cases are available for analysis Of these the two cases who survived for 5 days with death in bronchopneumonia on the 5th day are considered atypical, leaving 18 cases for analysis The postmortem findings in these were rather unspecific Glottal oedema was found in 6, pulmonary oedema, pulmonary emphysema or visceral (and pulmonary) congestion were found in 7, 8 and 11 cases respectively, petechial haemorrhages in skin, mucosa and/or serous membranes in 7 cases

Postmortem findings of possible importance in isolated cases were cardiac hypertrophy with slight myocardial fibrosis, slight atherosclerosis with cardiac hypertrophy, atheroma in descending branch of left coronary artery with severe narrowing of the lumen Two of the 19 autopsied cases had struma, none of them toxic

Histologic examination was only performed in 6 human cases (*Halperin*, *Wegelin*, author's first case)

Langer (1897) injecting bee venom intracutaneously in rabbits found a minute localised necrosis with granulocytes surrounded by oedema hyperaemia and some slight infiltration with lymphocytes *Kirshaw et al* (1949) observed cellular infiltration and local oedema surrounding minute muscular necrosis after wasp sting in a guinea pig

Halperin and *Wegelin*'s first two cases revealed only capillary hyperaemia oedema and minor haemorrhages and in some cases focal necroses In *Wegelin*'s last case (female, 39 years) the sting canal was beautifully demonstrated piercing the epidermis reaching the superficial layers of corium which contained a brown amorphous substance (venom?) The surrounding connective tissue was oedematous the small vessels and capillaries were dilated Slight haemorrhages with incipient destruction of red blood corpuscles were seen In this case as in the author's case 1 a particular detail was revealed i.e. focal necrosis of a sweat gland in the corium with surrounding engorged veins and capillaries

Toxicologic examination was only performed in the author's first case

DISCUSSION

Analysis of the few published cases of fatal stings from hymenoptera, including the author's 4 cases, revealed that the local symptoms were

(5) Author's first case of a 51-year old woman with adenomatous, non-toxic goitre, without previous disease.

So, from 34 cases of sudden death from hymenopter sting only 4 persons had a history of previous or preexisting diseases of possible importance. Of 20 autopsied cases 5 revealed postmortem findings of possible importance. In the whole material only two persons had goitre, which hardly support "the interesting connection with the thyroid gland" stressed by Thompson.

It is noteworthy, however, that the alleged heart and lung disease appeared to be only slight or inconspicuous, and in 30 cases the victim had either enjoyed a perfect health, or no information of preexisting disease was at hand. Although the significance of preexisting disease can hardly be denied, it appears that this can not be the explanation in the majority of the reported cases.

4. The fourth possibility of causal factors in the fatal cases as discussed by Wegelin is that of *anaphylactic shock due to hypersensitivity to bee and wasp venom in the victims*. Several authors (Wegelin, Swinny, Schenken et al., Marshall, Vueller, Parrish and others) and many important observations are in favour of this theory.

The symptoms in the fatal cases are quite typical of an anaphylactic shock, comparably, for instance, to the reaction following injection of foreign serum to sensitized individuals (Jex Blake 1942). As mentioned the hymenopter venom does contain proteins capable of inducing anaphylactic reactions. It is important therefore to consider how many victims of fatal stings had a history of previous stings and stings followed by systemic reactions.

Of the 34 reported cases positive information about one or more previous stings was given in 20 cases and in 12 of these severe reactions were reported. Only in one case the statement was given that the person had never been stung before, whereas in 13 reports did not contain information concerning this detail.

Of course a considerable percentage of people have been subject to stings of hymenoptera: some may not even remember previous stings, especially if these did not induce serious troubles, whereas stings with alarming reactions are more apt to be remembered. It is obviously often impossible to check the history concerning previous stings, in fatal cases take a sudden and rapid course. Considering these difficulties it appears to the author that the percentage of cases, in which a positive mentioning of previous stings is at hand, is quite conspicuous. Although the percentage of people stung in the general population is unknown, it appears fairly peculiar that so many of the victims had previous stings which might add to the evidence in favour of an anaphylactic pathogenesis. It is of interest too that 2 persons in this material had a history of *asthmatic allergy*.

One more fact points in favour of the anaphylactic theory, i.e., the fact that practically no children succumb to stings of bees and wasps.

It is difficult to reject this theory, but actually little if any proof of its correctness is at hand. The proof should of course be direct demonstration by means of a magnifying lens or in histologic sections, that the sting canal did enter a vein or capillary. Very few investigators, as mentioned, have performed histologic examinations on stings.

Neither these or the singular experimental observations revealed evidence of intravenous route of inoculation. The two cases in which sweat gland necrosis was observed (*Wegelin's* last, author's first case), however, might be considered as evidence of an indirect intracapillary network. The fact that children rarely succumb to bee or wasp stings furthermore weakens the theory of intravenous injection in fatal cases of stings as this should be expected to occur more often in children with thin epidermis. Further experiments might solve the interesting question concerning possible intravenous inoculation. So far, however, not a single reliable case of fatal intravenous inoculation is on record.

3. The third important factor stressed by *Wegelin* was that of *pre-existing serious disease*, notably of the heart or lungs. This might of course lower the resistance against the venom, as against other noxious influence.

Of the cases surveyed (Table 1 and 2) 11 persons were known always to have enjoyed a good health. In 17 cases nothing concerning previous health was stated, which might most probably indicate that no serious preexisting or previous illness was at hand. In 5 cases only, information concerning previous or preexisting disease was mentioned. The cases were: (1) *Hansen's* case (1921) of a 39-year old woman, who 12 years previously had uterine fibroids removed. She was reported to be otherwise healthy, and obviously no weight could be laid on this former operation. (2) *Alexander's* case (1938) of a 56-year old woman, who for some years had suffered from hypertension and cardiac disease. (3) *Hallas'* third case (1943) of a 46-year old man suffering from asthma since childhood, but without attacks for the last two years. (4) *Hallas'* fourth case (1943) of a 41-year old woman complaining since some years of slight heart-troubles, without serious symptoms. She also suffered from chronic leg ulcer. (5) Author's fourth case of a 67-year old woman, disabled from asthma-bronchitis, who succumbed in an asthmatic attack a few minutes after a sting.

In a further 5 cases autopsy revealed lesions, which might possibly have contributed to death. (1) *Halperin's* second case of a 46-year old man with cardiac hypertrophy, slight myocardial fibrosis and a pronounced non-toxic goitre, without information of previous disease. (2) *Halperin's* third case of a 52-year old man with slight general atherosclerosis and cardiac hypertrophy without information of previous disease. (3) *Wegelin's* third case (1948) of a 33-year old man with fatty infiltration of the liver without information of previous disease. (4) *Marshall's* case (1957) of a 52 year old man with coronary atherosclerosis with severe stenosis who had always enjoyed a good health.

has failed 10 to 20 ml 20 per cent solution of calcium gluconate should be given intravenously supplemented by 10 ml 10 per cent solution intramuscularly.

In shocked or unconscious patients infusions with ACTH and cortisone are advised (Williams 1951), and oxygen and artificial respiration might be considered.

Patients with sting in mouth, palate or throat must be carefully observed and if evidence of glottal oedema occurs, prophylactic tracheostomy must immediately be performed.

Antihistamines taken orally are slowly resorbed but given intravenously to shocked patients it has proved useful in some cases.

3 Prophylactic treatment. Mueller (1959) advises specific desensibilizing therapy to all patients with severe systemic reactions to stings or hypersensitivity to intracutaneous tests with whole extract of bees and wasps. Initial dose should be that giving "the initial positive test" which is then gradually increased to maintenance dose 0.2 to 0.3 ml of a 1 per cent solution. When cutaneous testing is not performed Mueller recommends an initial dose at the level of 0.05 of a 1:100 000 000 solution.

Great precaution must be undertaken during desensitizing. Adrenaline for injection should always be at hand as anaphylactic reactions may be provoked (For further details concerning desensitizing see Mueller).

In the outpatient clinic for allergic diseases Rigshospitalet Copenhagen desensibilizing treatment has been undertaken for several years (Bruun 1950). Preparations for this purpose, which are not on sale in Denmark at present ought to be more easily accessible, and other clinics should be encouraged to institute specific prophylactic therapy.

SUMMARY

Four fatal cases of stings from bees and wasps are reported. Three with complete postmortem examination. Survey on literature with listing of 30 previously reported cases is added.

Most fatal cases drabbed adults; the fatalities usually occurred within 30 to 60 minutes after the sting which was suddenly followed by cardio-pulmonary symptoms with shock and unconsciousness.

Lesions most often seen at autopsy were glottal oedema, pulmonary emphysema and oedema, visceral congestion and haemorrhages in skin, mucosa and serous membranes. Microscopy revealed the sting canal and localized necroses in sweat glands in two cases (Wegelin, author). A slight inhibition of serum cholinesterase was indicated in one of the author's cases.

It is concluded that the majority of deaths due to stings of bees and wasps are caused by anaphylactic shock. Only few fatal cases may be caused by mere local reactions, glottal oedema with suffocation from stings in the throat.

Children, of course, are very strongly exposed, they play in fields and gardens, where the insects are present, especially small children take less care than grown-up people do. Furthermore, children sometimes attract bees and wasps by eating sweets out-of-doors. If fatal reactions to the venom of hymenoptera were simply comparable to the effect of other poisons, small individuals should be expected to be less resistant, as the dosage per kilogram must be higher. In deaths from other poisonous animals and insects (snakes, scorpions and spiders) 41 per cent of all deaths occurred in the age group 0-19 years, whereas only 7 per cent of deaths from hymenoptera occur in the same age group (Parrish).

The fact that only two children are included in the cases reported (Table 1 and 2) points strongly in favour of the anaphylaxis theory, as mostly grown-up people have had "the chance" to develop anaphylaxis through previous stings.

The author thinks that the high percentage of previous alarming systemic allergic reactions to bee or wasp stings in many of the cases here reported is of great significance. Clinicians, who have patients with allergic manifestations to stings, should consider a specific desensitizing therapy. For the pathologist or forensic pathologist evaluating a case supposed to have died from hymenopter sting, information about allergic symptoms to previous events adds strength to his diagnosis in the actual case.

Suggestions for Therapy in Cases of Stings

1 *Local treatment* It is worth remembering that, contrary to wasps, bees when stinging do leave their sting in the skin of the victim. Removal of sting must be done with great care. It should be scratched away with a knife or a fingernail. Grasping with fingers or blunt forceps will probably inject more venom, as the poison sac usually is still attached to the sting. Local application of antihistamine ointment is beneficial, pain is usually almost immediately relieved, and further swelling of the affected part rarely occurs.

Treatment with ammonia water or other basic substances so popular among lay people, is useless, based on the erroneous belief, that the toxic substance in the venom is formic acid.

2 *Treatment of systemic reactions* Persons with previous or manifest severe allergic reactions should be treated immediately, as symptoms may rapidly proceed to a state of profound shock or even death. Adrenaline seems to be the most effective drug in treatment of anaphylaxis. 0.5-1.0 mg (0.5-1 ml of a 1:1000 solution) to a medium sized grown-up person should be injected slowly intravenously, and repeated if necessary.

Calcium, for instance 10 to 20 ml 10 per cent solution of calcium gluconate given intravenously is highly estimated by several authors. Hallay (1943) reports on cases in which calcium had immediate and miraculous beneficial effect. Marshall (1957) advises that if adrenaline

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Suggestions for therapeutic measures in severe cases include intravenous injections of adrenaline, calcium gluconate and maybe ACTH and cortisone. Specific desensibilization is advised.

In every supposed fatality to hymenoptera sting a medicolegal autopsy should be performed to elucidate the cause of death. This may have great economic consequences, as insurance companies must accept such deaths as accidents liable for compensation according to the workmen's compensation act, if the fatal sting has happened during work.

ADDENDUM

After this paper had been submitted for publication, Mann & Bates (1960) reported on 11 fatal cases of insect bites.

Mann G T & Bates, Jr H R. The pathology of insect bites—*STH Med J* 53 1399, 1960.

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By the light of the new therapeutic era for the dermatophyte infections the exact mycologic diagnosis is of more importance than ever. As for onychomycosis and dermatomycosis *Candida albicans* is the fungus next to the dermatophytes that most frequently has been reported as pathogen. Nevertheless there is another *Candida* species one ought to pay attention to, namely *Candida parapsilosis*. Lodder & Kreeger van Rij (18) point out in their taxonomy of yeasts that it is striking that many strains of *C. parapsilosis* are isolated from diseased nails and skin. This observation we will fully confirm. It is indeed remarkable how often *C. parapsilosis* is to be found in diseased nails and skin.

Another fungus, the mould *Scopulariopsis brevicaulis* has several times been described as cause of onychomycosis. In the present study we will describe one case of onychomycosis due to this fungus.

MATERIAL AND METHODS

The material consists of scrapings from diseased nails and skin of 131 patients. The specimens were partly taken directly from patients who were sent to the Institute from dermatologists and other physicians and partly the specimens were dispatched from physicians who had taken the samples themselves. The samples were examined by the following methods:

According to the methods of the Delft school

RESULTS AND DISCUSSION

The material from 38 of the 131 patients examined revealed no growth of fungi. The microscopy in direct preparations was negative in 36 of these cases. The nail scrapings from the other two who presumably had onychomycosis revealed abundant mycelium by direct microscopy. One of these had taken griseofulvin for some weeks. Whether the other patient had got some therapy or not, we do not know.

1. Dermatophytes

As shown in Table 1 dermatophytes were isolated from 53 patients. In the material from three of these patients we could not see fungal elements in potassium hydroxide preparations. Specimens from the other 50 patients appeared to be positive by direct microscopy.

Trichophyton rubrum was found in 37 cases, 21 women and 16 men. All of these with the exception of three patients who had skin lesions

responsible for onychomycosis. *T. rubrum* is the most prevalent species re-

MYCOLOGIC INVESTIGATION OF DISEASED NAILS AND SKIN IN 131 PATIENTS

By

SIGNY RØHRSTOL

Received May 61

In Norway we do not see the problem of dermatophyte infections in man in the same degree as in countries with more hot and wet climate. However, the occurrence of ring worm infections is not infrequent in this country. Thus Bjørnstad (1) in 1956 described 6 cases of deep trichophytosis in orientation sport runners. In 1957 Odgaard & Neess (2) also reported 3 cases of sport runners with deep trichophytosis. They found that *Trichophyton verrucosum* was the causal agent in these cases. Recently Lindquist (3) has given a survey of species of dermatophytes commonly causing ring worm in domestic animals. He points out that ringworm infections are wide spread in Norway, and he finds that *T. verrucosum* is the most frequent cause of ringworm in cattle, which is in good agreement with the findings in England and other countries. The author has also isolated the following two dermatophytes *T. verrucosum* and *T. mentagrophytes* several times from persons who had been in contact with infected animals, and *M. canis* from a child who had been in contact with infected cats and dogs.

Since griseofulvin has revolutionized the therapy of dermatophyte infections there have been reported several cases of tinea infections of man successfully treated with this antibioticum (4-16).

In the last decades of this century *Trichophyton rubrum* is the dermatophyte species that has been the main problem of ringworm in man. Thus Calnan (17) wrote in 1958:

"In company with many other European countries such as Germany, Holland and Switzerland, as well as the United States, we in Great Britain have experienced over the past decade a remarkable increase in the incidence of *Trichophyton rubrum* infections. It is likely that this infection has been endemic in China, Japan and the Far East for a long time, and that during the last 20 or 30 years it has spread progressively westwards over Asia and Europe, and eastwards to Australia and America."

In our laboratory we have realized that *T. rubrum* infections also in Norway are more common than generally assumed, especially those cases of typical chronic onychomycosis.

By the light of the new therapeutic era for the dermatophyte infections the exact mycologic diagnosis is of more importance than ever. As for onychomycosis and dermatomycosis *Candida albicans* is the fungus next to the dermatophytes that most frequently has been reported as pathogen. Nevertheless there is another *Candida* species one ought to pay attention to namely *Candida parapsilosis* Lodder & Kreeger van Rij (18) point out in their taxonomy of yeasts that it is striking that many strains of *C. parapsilosis* are isolated from diseased nails and skin. This observation we will fully confirm. It is indeed remarkable how often *C. parapsilosis* is to be found in diseased nails and skin.

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1. *Dermatophytes*

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Trichophyton rubrum was found in 37 cases: 21 women and 16 men. All of these with the exception of three patients who had skin lesions only had onychomycosis as the main problem. The fact that more than 75 per cent of the patients with positive mycologic findings were infected with *T. rubrum* and that nearly all of them had nail complications indicates that this fungus also in our country is the most prevalent species responsible for onychomycosis.

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registrate unless we should carry out mass examinations from various groups of people. It therefore is of little interest to tabularize the incidence of onychomycosis, *linea pedis*, *linea corporis* etc. in this material.

2. Yeasts

As already pointed out *Candida parapsilosis* was the *Candida* species that much against the expectation outnumbered *Candida albicans*. Since *C. albicans* has been considered as the most common pathogen among the *Candida* species and by many mycologists as the only one, this result is rather surprisingly.

Table 2 shows the yeasts isolated from 40 patients. The direct microscopy from the specimens issued in positive findings in 21 cases of which 12 also were infected with dermatophytes. Thus only in 9 of these patients the fungus was the proved infectious agent. Six of these patients was infected with *C. parapsilosis* and the other three with *C. albicans*.

Candida parapsilosis was found in 21 patients, 11 women and 10 men. As mentioned above the direct microscopy in potassium hydroxide preparations were positive in six of the cases with pure yeast cultures. Cultivated on the different media there were abundant growth of the fungus, so there should be no doubt that these six patients really were infected with *C. parapsilosis*. One of these = 30 year old man had a skin lesion on the right lower leg that he had for about two years. It was an isolated oval lesion about 4 to 10 cm in circuit with white macerated epithelium characteristic of skin moniliasis. Another man, 62 year old, was sent to the institute with a severe *T. rubrum* like infection. As usually it had started on the feet. All the toe nails were involved and both the footsoles had the fine scaling that one see in the typical *T. rubrum* infections. The fingernails on the right hand and the palm and fingers on this hand was hyperkeratotic with scaly patches. He was very much depressed by the situation as he had had his infection for many years. The direct microscopy from nails and skin scrapings showed abundant mycelium and spores and *C. parapsilosis* was cultivated in abundance. The four other patients with positive findings in direct preparations suffered from onychomycosis, one man and three women.

TABLE 2

	<i>Candida parapsilosis</i>	<i>Candida albicans</i>	Other <i>Candida</i> species	Other yeasts	Yeasts
Men	10	5	2	3	20
Women	11	4	1	4	20
Total	21	9	3	7	40

Trichophyton mentagrophytes was isolated from 8 patients, three women and five men. One of them was a stableman who was in daily contact with the guinea pigs and white mice in the stall of the institute, where some of the animals were infected with *T. mentagrophytes*. He had a typical ring-worm lesion on the left under-arm. The seven other patients had *tinca pedis* and two of these suffered from onychomycosis. Usually infections with *T. mentagrophytes* are of a milder type than those with *T. rubrum*. That this is true we also have realized. However one of the patients, a 43 year old man, had a severe infection with *T. mentagrophytes*. The infection had started with a simple foot ring-worm that gradually had involved the soles, the toe nails, the finger nails and at least the inside of fingers and the palms. When he came to the institute for examination he had suffered for years, and as indicated above the clinical picture was very like a *T. rubrum*-infection. After five months griseofulvin therapy he came back to control. He was then much better and he hoped he could stop therapy. The nails were however not yet cured and we got positive cultures both from toe nails and those from the hands.

TABLE 1

	<i>Tricho- phyton rubrum</i>	<i>Tricho- phyton mentagro- phytes</i>	<i>Tricho- phyton verru- cosum</i>	<i>Epidermo- phyton floccosum</i>	<i>Derma- tophytes</i>
Men	16	5	3	3	27
Woman	21	3	2	0	26
Total	37	8	5	3	53

Trichophyton verrucosum was found in five patients, two women and three men. They had all been in contact with domestic animals and had ring-worm lesions on the glabrous skin. None of them had onychomycosis.

Epidermophyton floccosum was isolated from three young men with *tinca pedis*. They were all from different parts of the country.

In this investigation the relation between the occurrence of the different dermatophyte species is not representative for the incidence of dermatophyte infections in the norwegian people. The specimens examined on our institute have to be a selected material because the patients who really suffer, physical and especially pschicall, first and foremost are doing what they can to get rid of their worries. They therefore go to their doctors who in the first instance communicate with our laboratory. Undoubtedly there are many people with *tinca pedis* and ring worm on the glabrous skin,—especially with *T. mentagrophytes*-, *T. verrucosum*- and *E. floccosum*-infections who have little or no trouble at all by their contaminations. Those people we are unable to

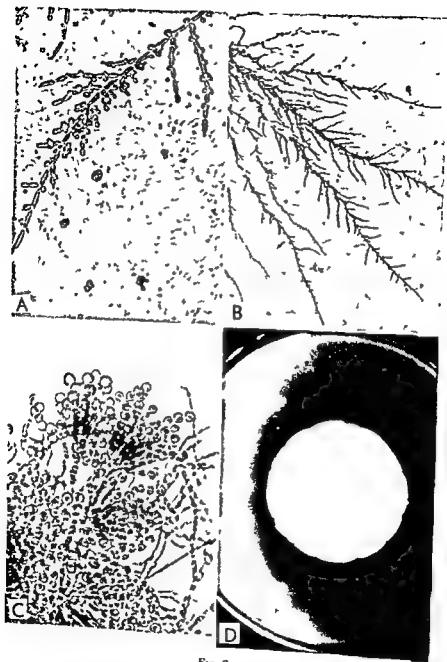


Fig 2

- A *Candida parapsilosis* 450 X Slide culture
 B *Candida parapsilosis* 100 X Slide culture
 C *Secularia* *breviscaulis* 450 X Slide culture
 D *Candida parapsilosis* Culture 21 days old on salmon agar

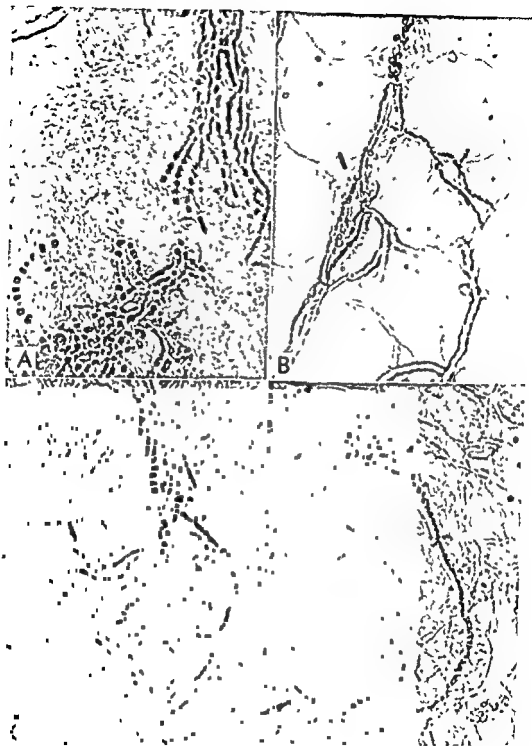


Fig 1

Direct microscopic preparations in 30 per cent potassium hydroxide 450 ×

- A *Candida parapsilosis* in nail
- B *Trichophyton rubrum* in nail
- C *Candida albicans* in nail
- D *Scopulariopsis brevicaulis* in nail

3 Other Fungi

Scopulariopsis brevicaulis was found to be pathogen in one case of onychomycosis. The patient was a 30 year old man with infection on the fingernails of both hands. The edges of the nails were ragged and deformed and the nails had the cheesy debris characteristic of *Scopulariopsis* infected nails.

As for the appearance of single colonies of moulds or yeasts we have taken it for granted that these have been saprophytes. Thus we have not taken notice of such contaminations in this investigation.

4 Mixed Infections

From one of the specimens dispatched to us there were growth of the following two dermatophytes *Trichophyton rubrum* and *Trichophyton mentagrophytes*. The material proceeded from a young girl with a severe foot ringworm with onychomycosis.

As already mentioned 19 of the patients with dermatophyte infections were also infected with yeasts. Seven of these were *T. rubrum* infections combined with *C. parapsilosis*, three *T. rubrum* with *C. albicans*, one *T. mentagrophytes* with *C. albicans* and one *T. rubrum* with *C. guilliermondii*. The latter case was a 37 year old woman. She had suffered from onychomycosis of the hands for several years. The first time we examined her nails we got abundant growth of *Candida guilliermondii* only. One month later we moreover got growth of *T. rubrum* especially from the palm of the right hand that were infected as well. Seven months later we still isolated *C. guilliermondii* and *T. rubrum* from the nails and palm of this patient.

SUMMARY

Skin and nail specimens of 131 patients were examined with regard to mycotic infections. There were negative findings from 36 of these patients. 95 patients were infected with dermatophytes. *Trichophyton rubrum* was found in 37 cases of which 34 suffered from —

as *Tricho*

phyton etc.

Yeasts were isolated from 40 patients. Interesting is the high incidence of *Candida parapsilosis* which was isolated from 21 patients while *Candida albicans* was found only in 9 cases. Since *C. albicans* has been considered as the most common pathogen among the *Candida* species and by many mycologists as the only one, this result is remarkable.

Grateful acknowledgement is made to Miss Annaljelgerud and Mrs. Else Klogetved for the technical assistance.

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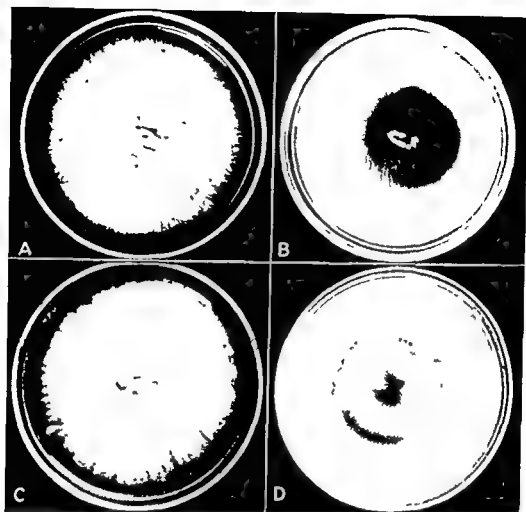


Fig 3

- A *Epidermophyton floccosum*, Sabouraud agar
 B *Trichophyton rubrum*, corn meal agar
 C *Scopulariopsis brevicaulis* Sabouraud agar
 D *Trichophyton rubrum* corn meal agar

Candida albicans was isolated from 9 patients, five women and four men. In the samples from three of these there were positive findings in potassium hydroxide preparations, two women and one man, all with onychomycosis.

Concerning patients with skin and nail lesions with negative findings by direct microscopy in potassium hydroxide preparations and with abundant growth of yeasts from the samples, it is very difficult to decide whether the fungus only is a saprophyte or a pathogen. The presence of yeasts as saprophytes all over the world and none the less on the human being, is obvious. We therefore have to be very cautious with the diagnosis of fungus infections in such cases. Most likely the yeasts should be considered as saprophytes.

MECHANISM OF EXPERIMENTAL TUMORIGENESIS

II Sulfhydryl Groups, Disulfide Bonds, and Birefringence in Mouse Epidermis after Exposure to Dipole Type Tumor Promoter and Carcinogen

By

KAI SETÄLÄ, OSMO A. AYRÄPÄÄ and EINO ERKKI NISKANEN

Received 25 x 61

The hyperplastic states caused by carcinogens and by dipole-type tumor promoters in the epidermis of a skin-tumor-resistant mouse strain have been found to differ in all the features studied so far, being in several ways each other's opposites [reviewed in (1,2)]. Ever since we began to use nonpolar-polar agents in the study of carcinogenesis (*e.g.*, 3-5) we have thus tried to separate the effect of the tumor promoting agent from the total complex of the carcinogenetic process and then to analyze what is left over.

The purpose of this study is to demonstrate on a large experimental material that the behavior of histologically demonstrable protein-bound sulfhydryl groups, disulfide bonds, and birefringent material also essentially depends on whether the skin of the back of the mouse of a tumor-resistant strain is exposed to carcinogens or to dipole-type tumor-promoting agents.

MATERIALS AND METHODS

The general experimental procedures appear in the earlier papers of this series. Male and female mice of this laboratory's randomly bred skin tumor resistant RA strain were used.

The carcinogens were ¹⁴C-³H-

cholanthrene (MCA) for

local exposure to DMBA

employed as solvents. I

For oral administration the solvent was

undiluted Carbowax 400 from Carbide and Carbon Chemicals Co. New York.

The dipole type technical (t) and laboratory synthesized (s) agents of the Span and Tween types were employed in undiluted form as 0.18 M aqueous solutions.

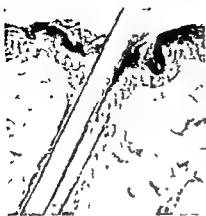
Figure 1 shows

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PLATE 1



or dispersions, or 0.0066 M acetone solutions. The characteristics of the compounds appear in (12).

Skin biopsies from the back of the mice were taken from 5 different animals in all series on days 2, 6, 10, 16, 30 and 60 always between 8 and 9 AM. At each time 5 successive sections were cut at 10 μ : the 1st was stained with hemalum and eosin for orientation and control, the 2d was left unstained for orientating polarization optical examination, the 3rd for qualitative histochemical demonstration of protein bound sulfhydryl (-SH) groups, the 4th for identification of disulfide (-S-S-) bonds and the 5th for demonstration both of -SH groups and -S-S- bonds. The techniques suggested in (6-8) were employed. The qualitative reliability and sources of error appear in (6-8). The phase of the hair follicular cycle was observed. Preparations from different experiments were photographed side by side at one sitting using the same film material and the same exposure values. All experiments were repeated several times and identical results were obtained.

In routine experiments birefringence was studied with a modified Leiss polarization microscope equipped with nicol prisms and a planacromat III/100/1.25 Pol (for theory and nomenclature see (9,10)). All biopsy specimens were analyzed from carefully deparaffined unstained sections (2d sections) that were mounted in canada balsam.

To determine the exact localization of birefringence, another part of each specimen was embedded in methacrylate and trimmed into pyramidal form [technique in (11)]. Thin sections were cut with the ultramicrotome in two planes at right angles to each other: the first set was cut sagittally to the dermoepidermal junction (S section), and the second set parallel to the cutaneous surface as in our previous electron microscope studies (P section) [e.g. in (11-13)]. Both sections were first photographed under the electron microscope and then under the polarization microscope (the results obtained with this technique will be published elsewhere).

The Experiments Consisted of

1 Normal controls

2 Mice treated with dipole type agents (continuous local exposure of the back of the mouse 6 and 12 times a week to undiluted and ∞ 0.18 M aqueous solutions of t and s Tween 60 (polyoxyethylene sorbitan monostearate) and t Tween 40 (polyoxyethylene sorbitan monopalmitate), 6 times a week to ∞ 0.18 M aqueous solutions

PLATE 1

Fig 1 Mouse No 20957 male normal skin of back -SH preparation. Hair almost colorless (negative histochemical reaction), cytoplasm of nucleated cells of III and outer root sheath pink (moderate reaction), nuclei almost colorless as in Figs 2 through 6. Stratum corneum blue violet (very intense reaction). Hair follicular cycle in Figs 2 through 5 telogen.

Fig 2 Mouse No 21060 female 16 mg of DMBA orally 5 times 7th day -SH preparation. Irregular hyperplasia of III and outer root sheath (cytoplasm of nucleated cells bright red violet (intense reaction), keratotic plug within dilated follicular opening strongly blue violet like stratum corneum).

Fig 3 Same normal animal and same hair follicle as in Fig 1 -SH preparation. Middle hair and sebaceous cells almost colorless but keratogenous zone blue violet. Cytoplasm of nucleated cells of outer root sheath pink.

Fig 4 Same carcinogen treated animal and same hair follicle as in Fig 2 -SH preparation. Cytoplasm of nucleated cells of hyperplastic outer root sheath bright red violet. Keratogenous zone strong blue violet.

Fig 5 Mouse No 18371 female about 80 mg of undiluted t Tween 40 locally 6 times a week 30th day -SH preparation. Regular III hyperplasia. Stratum corneum very thick. Cytoplasm of nucleated cells pink. Stratum corneum partly bright red violet partly blue violet.

Fig 6 Mouse No 20698 male 60 γ of MCA locally 6 times a week 30th day -SH preparation. Irregular III hyperplasia. Stratum corneum thick and irregular. Cytoplasm of nucleated cells [e.g. violet] stratum corneum blue violet. Reactivity essentially differs from that in Fig 5. Hair follicular cycle not definable.

Figs 1-6 original magnification $\times 530$

corneum depicted a very intense reactivity. The cytoplasm of the nucleated cells showed a moderate concentration of $-SH$. Thus the histochemical appearance in experiments with continuous exposure to 60 γ of DMBA and MCA differed essentially from normal (Fig. 1) as well as from that obtained after continuous local treatment with 80 mg of Tween type tumor promoters (cf. Fig. 5).

In preparations from mice given carcinogen orally (Figs. 2 and 4), the distribution and reactivity of $-SH$ was the same as in experiments with local exposure. The concentration of $-SH$ had distinctly increased from normal (cf. Figs. 1 and 3), and was greater than in mice treated with tumor promoters. Control experiments with repeated doses of Carbowax 400 revealed normal histochemical reactivity of $-SH$.

Benign keratinizing skin papillomas—In all preparations, the behavior of $-SH$ was identical. The superficial keratogenous mass which was composed of numerous scales stained somewhat unhomogeneously (Fig. 12). In some instances it turned pink, in other cases it had a deep violet color. The cytoplasm of the nucleated papilloma cells showed a moderate reactivity. Nucleated cells in the surrounding nonpapillomatous epidermis behaved similarly. Within the papilloma, the zone between the superficial horny mass and the nucleated cells revealed a very intense reactivity. There was no such strongly reactive zone in the surrounding nonpapillomatous epidermis. This zone terminated suddenly at the border between the papilloma and the surrounding epidermis.

S-S Bonds

Normal epidermis—The behavior of the reactive materials was, in principle, like that previously reported in normal mouse skin (8). The stratum corneum and the outer layer of the follicular openings had a weak shade of blue violet revealing a moderate presence of $-S-S-$ (Fig. 7). The cytoplasm of the nucleated cells of the IFF and outer root sheaths showed a low, though distinct concentration of $-S-S-$. The hair shaft cuticle and medulla showed high amounts of $-S-S-$ bonds.

In the skin of mice given carcinogen correspondingly longer and the color more intense. In other respects the reactivity was practically independent of the hair follicular cycle.

Effect of dipole type agents—T and S products caused identical changes.

In the skin of mice given carcinogen correspondingly longer and the color more intense. In other respects the reactivity was practically independent of the hair follicular cycle. The cytoplasm of the nucleated cells in the regularly hyperplastic IFL, as well as within the

and 0.0066 *M* acetone solutions of α -glyceryl-1-stearate 2:1 bis polyoxyethylene ether and α -glyceryl-1,2-distearate 3 polyoxyethylene ether, and to 0.0066 *M* acetone solution of glyceryl-1,2-distearate, which are dipole-type tumor promoters, as well as 6 times a week to ∞ 0.18 *M* aqueous dispersions of t and s Span 60 (sorbitan monostearate) and t and s Span 20 (sorbitan monolaurate), and ∞ 0.19 *M* aqueous solutions of t and s Tween 20 (polyoxyethylene sorbitan monolaurate), which are weak tumor promoters or agents lacking this property (14).

3 Mice treated with carcinogens. Continuous local exposure of the back of the mouse 4 times a week to 60 γ of DMBA and MCA, a single gastric instillation of 16 mg of DMBA and MCA in 0.15 ml of undiluted Carbowax 400, 5 similar instillations on successive days as well as 0.15 and 0.30 ml of Carbowax 400 on 5 and 10 successive days without carcinogen.

4 Numerous histologically verified typical benign skin papillomas that were provoked with the two stage technique using various dipole agents as tumor promoters.

RESULTS

Protein-Bound —SH Groups

Normal epidermis—The 1st sections were examined and cases showing deviation from the normal were excluded. In preparations processed for identification of —SH groups, the stratum corneum was blue-violet, i.e., showed a high concentration of protein-bound —SH (Fig. 1). The cytoplasm of the nucleated cells of the interfollicular epidermis (IFE) [defined, e.g., in (1, 11–13)] and outer root sheaths was pink. This suggests a moderate amount of —SH. The nuclei were almost colorless, which indicates an absence of —SH. The hair cuticle, at its full length, reacted moderately. The distal and middle parts of the hair medulla were not reactive. The keratogenous zone of the hair showed a very intense reactivity (Fig. 3). The blue-violet material within the keratogenous zone was fan-shaped, the length of this zone depended on the hair follicular cycle. These results agree with those encountered previously with this technique (6–8) and with those obtained biochemically in thick epidermis (15).

Effect of dipole-type agents—T and s agents caused identical features.

In experiments with Tween-type agents, the distribution of —SH was found to be nearly the same both in regularly hyperplastic IFE (Fig. 5) and in the well-preserved pilo sebaceous apparatus irrespective of the hair follicular cycle, just as in the normal controls. The thick, but rather regularly layered stratum corneum showed a very intense histochemical reactivity, though the stainability varied somewhat from one place to another. The cytoplasm of nucleated cells in the hyperplastic IFE displayed a weak —SH concentration.

In experiments with Span-type agents, the distribution of —SH was about the same as in normal epidermis. The exception was α -glyceryl-1,2-distearate, which is a medium-powered tumor promoter (14) causing epidermal hyperplasia (16). It behaved like the Tween-type tumor promoters.

Effect of DMBA and MCA—In experiments with local application of carcinogens (Fig. 6), the unevenly thickened and deranged stratum

corneum depicted a very intense reactivity. The cytoplasm of the nucleated cells showed a moderate concentration of $-SH$. Thus the histochemical appearance in experiments with continuous exposure to 60 γ of DMBA and MCA differed essentially from normal (Fig 1) as well as from that obtained after continuous local treatment with 80 mg of Tween type tumor promoters (cf Fig 5).

In preparations from mice given carcinogen orally (Figs 2 and 4), the distribution and reactivity of $-SH$ was the same as in experiments with local exposure. The concentration of $-SH$ had distinctly increased from normal (cf Figs 1 and 3) and was greater than in mice treated with tumor promoters. Control experiments with repeated doses of Carlinox 400 revealed normal histochemical reactivity of $-SH$.

Benign keratinizing skin papillomas—In all preparations the behavior of $-SH$ was identical. The superficial keratogenous mass which was composed of numerous scales stained somewhat unhomogeneously (Fig 12); in some instances it turned pink, in other cases it had a deep violet color. The cytoplasm of the nucleated papilloma cells showed a moderate reactivity. Nucleated cells in the surrounding nonpapillomatous epidermis behaved similarly. Within the papilloma the zone between the superficial horny mass and the nucleated cells revealed a very intense reactivity. There was no such strongly reactive zone in the surrounding nonpapillomatous epidermis. This zone terminated suddenly at the border between the papilloma and the surrounding epidermis.

§ 5. Bonds

Normal epidermis—The behavior of the reactive materials was in principle like that previously reported in normal mouse skin (8). The stratum corneum and the outer layer of the follicular openings had a weak shade of blue violet revealing a moderate presence of $-S-S-$ (Fig 7). The cytoplasm of the nucleated cells of the IFF and outer root sheaths showed a low, though distinct concentration of $-S-S-$. The hair shaft cuticle and medulla showed high amounts of $-S-S-$ bonds.

In the case of hair length during the anagen phase the keratogenous zone appeared correspondingly longer and the color more intense. In other respects the reactivity was practically independent of the hair follicular cycle.

Effect of disulfide type agents—T and S products caused identical changes.

In experiments with Tween type agents the most superficial lamellae of the thickened stratum corneum revealed very intense and the lower lamellae intense reactivity (Fig 9 cf Fig 7). The cytoplasm of the nucleated cells in the regularly hyperplastic IFF as well as within the

well-preserved outer root sheaths showed a weak, though clearly distinguishable reactivity for -S-S- Just as in the untreated controls, the hair had a normal distribution and amount of -S-S- bonds

In experiments with Span-type agents, no essential deviation from normal controls was evident The skin exposed to *s* glyceryl-1,2 distearate had a distribution of -S-S- similar to that in experiments with Tween-type agents

Effect of DMBA and MCA—In experiments with locally applied DMBA and MCA, the strongly thickened and severely deranged stratum corneum was in most places colorless or almost colorless (Figs 8 and 10) This indicates an absence of -S-S- bonds At places (Fig 8) some keratin squame, or horny pearls, or both revealed a low concentration of -S-S- The cytoplasm of the nucleated cells had either a slightly beige-pink or almost colorless appearance Changes brought about by local exposure to DMBA developed faster and were more severe than those caused by MCA

In preparations from mice given DMBA and MCA orally, changes were essentially the same as above The stratum corneum and the cytoplasm of the nucleated cells showed a weak reactivity Preparations from controls with repeated high doses of Carbowax 400 were normal

Benign keratinizing skin papillomas—In all preparations the distribution of -S-S- was the same The superficial keratogenous mass stained somewhat unhomogeneously from pink to strong violet (Fig 11) The cytoplasm of the nucleated papilloma cells showed a weak reactivity

PLATE 2

Fig 7 Same normal animal and same hair follicle as in Fig 1 -S-S- preparation Hair bright red violet (intense reaction), cytoplasm of nucleated cells of III and outer root sheath pale pink violet (weak reaction), nuclei almost colorless (negative reaction) as in Figs 8 through 12 Stratum corneum weakly blue violet (moderate reaction) Hair follicular cycle telogen

Fig 8 Mouse No 20718 male 60 y of DMBA locally 8 times a week 30th day -S-S- preparation High degree irregular III hyperplasia, stratum corneum thick and deranged Cytoplasm of nucleated cells slightly beige pink or almost colorless some horny squame and a horny pearl pale pink violet Hair follicular cycle not definable

Fig 9 Same animal as in Fig 5 About 80 mg of undiluted *t* Tween 40 locally 6 times a week 30th day -S-S- preparation Cytoplasm of nucleated cells beige pink superficial lamellae of thickened stratum corneum strongly blue violet lower lamellae bright red violet Hair follicular cycle telogen

Fig 10 Same animal as in Fig 6 60 y of MCA locally 6 times a week 30th day -S-S- preparation Cytoplasm of nucleated cells beige pink stratum corneum slightly pale pink Remnants of hair bright red violet Hair follicular cycle not definable

Figs 7-10 original magnification $\times 530$

Fig 11 Mouse No 16410 male regularly built typical keratinizing skin papilloma provoked by two stage technique 10th week -S-S- preparation Superficial horny scales pink like cytoplasm of nucleated cells nuclei colorless Zone between horny mass and outer nucleated cells strongly blue violet

Fig 12 Same tumor and same place as in Fig 11 -S-S- preparation Superficial horny scales slightly brownish pink cytoplasm of nucleated cells red Zone between horny mass and nucleated cells blue violet

Figs 11-12 original magnification $\times 85$

PLATE 2



8



10



12

The zone between the superficial keratogenous mass and nucleated cells which showed strong stainability in $-SH$ preparations had a distinctly higher concentration of $-S-S-$ as well. Similarly, this $-S-S-$ reactive zone terminated abruptly at the border between the papilloma and the surrounding epidermis. The results were reproducible.

Preparations processed for simultaneous demonstration both of $-SH$ and $-S-S-$ revealed no features deviating from those in preparations stained for separate identification of $-SH$ groups and $-S-S-$ bonds. The same was true of preparations from normal skin as well as of those obtained in experiments with dipole-type agents and with carcinogens.

Birefringent Material

Normal epidermis—Within the basal cells the pattern of anisotropy varied essentially with the plane and direction of the section.

In serial S-sections double refraction appeared as delicate, slightly archlike striations at almost right angles to the cutaneous surface (Fig. 13). There were alternating anisotropic and isotropic zones. Birefringence was located either in the paramembraneous area at the cytoplasmic periphery or pressed against the perinuclear halo of cytoplasmic organelles. There were also a few delicate double refracting bundles extending from the lower poles of the nuclei to the dermo-epidermal junction. Thus when viewed sagittally, the bundles of anisotropic material correspond to Herxheimer's spirals [(17, 18), see also Figs. 1 and 2 in (17)].

In serial P-sections through the whole height of the basal cells, anisotropic material appeared in the perinuclear halo or paramembraneously.

PLATE 3

Photomicrographs 13 through 15 and 17 through 28 are from unstained sections in polarized light.

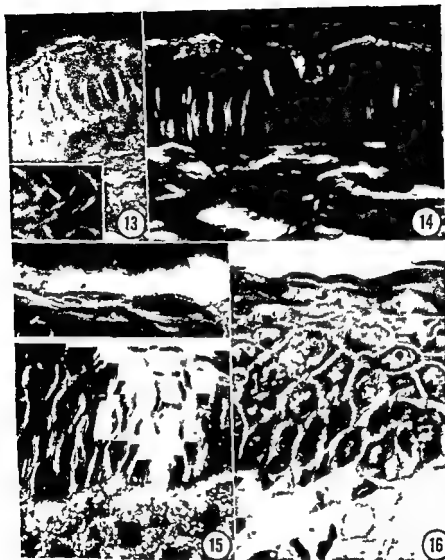
Fig. 13 Mouse No. 9595 male S section from IFT of back of normal mouse. Double refracting material consisting of fine, parallel, archlike bundles of birefringent material arranged perpendicular to cutaneous surface. Anisotropy in basal cells oriented along their longitudinal axis. Intercellular bridges show distinct double refraction. Order of anisotropy about same as in normal IFT. Hair follicular cycle anagen.

Fig. 14 Mouse No. 9897 male S section 0.0066 M acetone solution of s glyceryl 1 distearate (Spin 60 type agent) locally 6 times a week 30th day. IFT slightly thicker than normal. Order of birefringence in nucleated cells increased. Hair follicular cycle anagen.

Fig. 15 Mouse No. 17386 male S section ~ 0.18 M aqueous solution of s glyceryl 1,2 distearate 3 polyoxyethylene ether (Tween 60 type agent) locally 6 times a week 60th day. Stratum corneum thick and coarsely lamellated containing large amount of birefringent material partly cross cut. Anisotropy in basal cells perpendicular to cutaneous surface in differentiating cells orientated along their longitudinal axis. Intercellular bridges show distinct double refraction. Order and amount of anisotropy about same as in normal IFT. Hair follicular cycle anagen.

Fig. 16 Same specimen and same place as in Fig. 15 after stained with hemalum and eosin. This figure confirms localization of birefringent material.

Figs. 13-16 original magnification $\times 1120$.



as concentric profiles around the nuclei. There was a distinct isotropic zone between the anisotropic material and the nuclear interface. When the revolving stage of the polarization microscope was rotated, the birefringence appeared in a continuous manner. At places double refracting material touched the cell membrane. Serial P-sections revealed that the annular birefringence continued from the apex of the basal cells down to the dermoepidermal junction. Thus instead of the conventional, basally-apically oriented Herxheimer's spirals only, there exists a complicated, three-dimensional system of birefringent molecular chains in the cytoplasm of the basal cells surrounding the nuclei like a woven basket. The same cytoplasmic arrangement of anisotropic material was seen in differentiating cells.

The localization of the double refracting material in the hair as well as its intensity and dependence on the hair follicular cycle corresponded to those reported in the literature.

Effect of dipole-type agents—In experiments with Tween-type agents, the most conspicuous feature was that the localization and general arrangement of the anisotropy both in the regularly hyperplastic IFT and in the well-preserved pilo-sebaceous apparatus were regular or almost regular. The thickened and coarsely lamellated stratum corneum contained a large amount of brilliant double refracting material. In S-sections, anisotropy appeared either as spotlike granules (cross-cut, Figs 15 and 25) or almost parallel lamellae (longitudinally cut, Fig 26). In P-sections birefringent material appeared as highly brilliant bundles arranged in a comparatively regular pattern (Fig 21). The stratum granulosum was thicker than normal and contained a varying amount of anisotropic spots.

In the nucleated cells of hyperplastic IFT, as in those of normal IFT, the appearance of double refraction depended on the direction of the

PLATE 4

Fig 17 Mouse No 7191 male S section. About 80 mg of undiluted Tween 60 locally 6 times a week, 30th day. Hair shaft slightly distal from keratogenous zone. Structure well-preserved. Hyaloid membrane distinctly birefringent. External root sheath isotropic. Hair cuticle intensely anisotropic, birefringent material in hair cortex regularly oriented into fibrils. Hair follicular cycle: anagen.

Fig 18 Mouse No 20812 male S section. 60 γ of DMBA in acetone locally 6 times a week, 16th day. Hair shaft slightly distal from keratogenous zone. Structure severely deranged. No defined layers (cf Fig 17). Hair shaft contains a keratin cyst and compact birefringence. External root sheath reveals irregular birefringent material. Hair follicular cycle: anagen.

Fig 19 Same animal and same hair as in Fig 17 bulb. Structure well preserved. External root sheath isotropic. Henle's layer strongly birefringent. Internal root sheath depicts a few keratohyaline granules. Huxley's layer shows scanty birefringence. Cells of cuticle isotropic. Hair cortex composed of regularly layered birefringence.

Fig 20 Same animal and same hair as in Fig 18 bulb. Structure severely deranged. No defined layers (cf Fig 17). No distinct keratogenous zone. Some matrix cells show strong anisotropy.

Figs 17-20 original magnification $\times 1120$.



plane of section. In S-sections, birefringence in the basal-type cells appeared archlike nearly perpendicular to the dermoepidermal junction (Figs 15, 25 and 26). In differentiating cells, the anisotropy was arranged streaklike perinuclearly or paramembranously along the longitudinal axis of the cells (*e.g.*, Figs 15 and 16). The amount of double refraction was of about the same order as that in the basal cells of untreated controls (Fig 13). Also in P-sections through the level of nucleated cells (Fig 23), the anisotropy was arranged similarly to that in corresponding sections of the basal cells in untreated IFL: the nuclei were encapsulated within a birefringent basket. The intercellular bridges which developed in the mouse epidermis as a result of exposure to dipole-type tumor promoters showed strong birefringence both in S-sections (Figs 15 and 16) and in P-sections (Fig 23). The anisotropy in the well-preserved hair was regular and its position natural (Figs 17 and 19) and independent of the efficacy of the treatment.

In experiments with Span-type agents, the cytoplasm of the nucleated cells showed an amount of birefringence (Fig 14) higher than that in normal controls (*cf.* Fig 13).

Effect of DMBA and MCA—A severe derangement in the localization of anisotropy was conspicuous. Exposure to DMBA brought about more extensive changes than treatment with MCA. The alterations increased with the dose and the frequency of the treatment. The irregularly thickened stratum corneum had a birefringence whose degree and arrangement varied considerable from one place to another. In S-sections the double refraction appeared as uneven and deranged layers or lamellae (Fig 27). In P-sections (Fig 22), the orientation of anisotropy was disturbed, and fragmented and deranged bundles of brilliant birefringent material appeared at right angles to each other. The stratum granulosum had irregularly arranged anisotropic spotlike granules of varying sizes.

PLATE 5

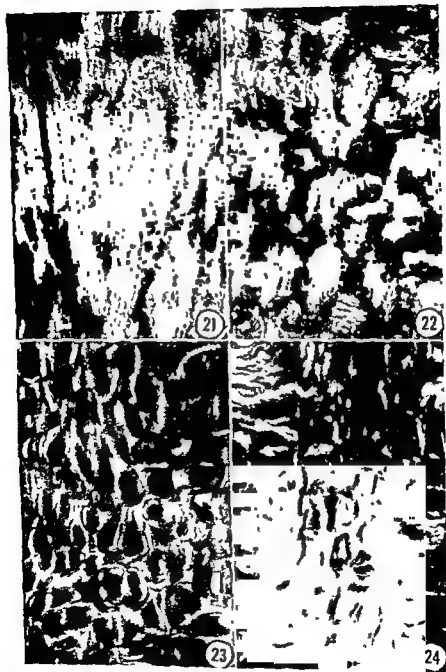
Fig 21 Mouse No 23710 male. P section. About 80 mg of undiluted t. Tween 60 locally 6 times a week, 30th day. Stratum corneum. Birefringent material arranged into bundles. Hair follicular cycle: telogen.

Fig 22 Mouse No 23612 male. P section. 60 γ of DMBA in liquid paraffin locally 8 times a week, 30th day. Stratum corneum. Orientation of birefringent material deranged; double refraction appears fragmented in zigzag fashion (*cf.* Fig 21). Hair follicular cycle: telogen.

Fig 23 Mouse No 23713 male. P section. About 80 mg of undiluted t. Tween 60 locally 6 times a week, 30th day. Upper layers of nucleated cells in hyperplastic IFL. In perinuclear halo or paramembranously almost continuous annular zone of birefringent material (*see text*). Intercellular bridges intensely birefringent (*cf.* S sections in Figs 15, 25 and 26). Hair follicular cycle: telogen.

Fig 24 Mouse No 23618 male. P section. 60 γ of DMBA in liquid paraffin locally 6 times a week, 30th day. Upper layers of nucleated cells in irregularly hyperplastic IFL. Deranged course and arrangement of cytoplasmic anisotropy. Amount of birefringent material varies from cell to cell. Hair follicular cycle: not definable.

Figs 21-24 original magnification $\times 1,120$



In the nucleated cells of the irregularly hyperplastic IFE, the birefringence varied between different cells and areas even in the same section. Some cells were nearly devoid of anisotropy, in others there was an increased amount of irregularly arranged double refracting material (e.g., Fig 27). This unevenness was evident both in the S-sections (Fig 27) and in the P-sections (Fig 24). Exposure to DMBA and MCA caused development of a ribbon-like, "sticky" connection between parental and daughter cells which is evident also in electron microscopy (1, 13, 16, 19). Fig 27 is from a S-section. Irregular, cablelike bundles of anisotropic fibrillar profiles are seen that extend from the region of the lower pole of the nuclei to the dermoepidermal junction. In P sections there was an increased amount of irregular annular birefringence arranged concentrically with the cellular and nuclear interfaces (Fig 24, cf Fig 23 from experiments with Tween) and appearing paramembranously, and sometimes perinuclearly as well, in the peripheral cytoplasm of some of the nucleated cells. The irregularity of the anisotropy in the severely injured hair (Figs 18 and 20) was evident.

Benign keratinizing skin papillomas—The keratogenous mass on the outer surface of the papilloma in Fig 28, i.e., a structure corresponding to the stratum corneum in normal and hyperplastic IFE, revealed a large amount of well-oriented lamellae of brilliant anisotropy. The same was true of the keratogenous material lining the horny cysts within the papillomas. In S-sections, the deeper lamellae nearest to the outer nucleated cells were more closely attached to each other than the superficial ones. These two parts of the keratogenous mass were seen also in preparations processed for identification of —SH and —S—S— (cf Figs

PI ATF 6

Fig 25 Mouse No 7229 male S section ~ 0.18 M aqueous solution of Tween 60 locally 12 times a week 30th day Stratum corneum thick amount of birefringent material varies anisotropic fibrils mainly cross cut. In regular hyperplastic IFE birefringence evenly distributed, orientation of anisotropic material normal Hair follicular cycle anagen.

Fig 26 Mouse No 20587 female S section ~ 0.18 M aqueous solution of Tween 60 locally 6 times a week 30th day Stratum corneum thick regularly lamellated intense birefringence intracytoplasmic double refraction oriented perpendicularly to cutaneous surface as in normal IFE (cf Fig 13) Hair follicular cycle telogen.

Fig 27 Mouse No 9126 male S section 60 γ of DMBA in acetone locally 11 times a week 30th day Stratum corneum coarsely lamellated birefringent material mainly parallel with cutaneous surface. Birefringence in cytoplasm of nucleated cells in IFE irregularly distributed a huge cell in sticky connection with dermoepidermal junction. Hair follicular cycle not definable.

Fig 28 25 '77 original magnification $\times 1120$

Fig 29 Mouse No 16411 male S section Regularly built typical keratinizing skin papilloma produced by two stage technique 36th week keratinous mass intensely birefringent cytoplasm of nucleated cells contains about normal amount of anisotropy (cf Figs 11 and 12 SH and SS preparations) Hair follicular cycle in surrounding nonpapillary matous IFE telogen. Original magnification $\times 170$



11 and 12) These structures were similarly lacking in the surrounding nonpapillomatous epidermis. In S-sections the birefringence in the cytoplasm largely resembled that observed in the hyperplastic IFI in experiments with dipole-type tumor promoters. In the basal-type cells, anisotropy appeared archlike at the cell periphery. In differentiating cells, it was directed along the longitudinal axis of the cells. In P-sections the birefringence was seen to be perinuclearly or paramembranously concentric with the cellular and nuclear interfaces. The intercellular bridges had a distinct birefringence.

DISCUSSION

It was shown that in a skin-tumor-resistant mouse strain, the effects of DMBA and MCA on the distribution and behavior of histochemically demonstrable sulfhydryl groups and disulfide bonds as well as on birefringence were essentially different from, and in several respects antagonistic to, those of dipole-type tumor promoters. Together with our earlier data (*e.g.*, 1, 2, 12-14, 20-23) these observations support the view that the effect of carcinogens is essentially different from that of dipole-type tumor promoters.

Histochemical studies showed that in the epidermis of normal mice, -S-S- bonds appear in the cytoplasm of undifferentiated basal cells (*cf.* 15). Examination in polarized light of sections cut serially at right angles to each other revealed a basketlike cytoskeleton arrangement woven of anisotropic molecular chains in the cytoplasm of the basal cells [the "keratinization organelle" (31)]. Herxheimer's spirals (17, 18), as well as tonofibrils and tonofilaments in electron microscopy (unpublished), form only that part of this basket which appears in sections cut sagittally to the dermoepidermal junction. The system as a whole is three-dimensional. In sections cut parallel to the cutaneous surface, another part of this cytoskeleton appears as concentric perinuclear rings. Keratinization was shown to proceed in close connection with this system. Previously it has been shown that the basal cells of the epidermis of skin-tumor-resistant mice usually lack the double membranes of the endoplasmic reticulum (11).

Our previous experiments with technical and laboratory-synthesized dipole-type tumor promoters have shown that the ultrastructure of the cytoplasm and its derivatives in the epidermal daughter cells maintains its undifferentiated features for a long time (12, 16). At the same time keratinization is secondarily delayed, but occurs ultimately, and the cells disintegrate (12). Now we have shown that exposure of the mouse skin to these agents does not essentially change the cytoplasmic distribution and amount of histochemically demonstrable -SH and -S-S- (Figs. 5 and 9). Similarly, the stratum corneum contained -SH and -S-S- as in the normals. The capsule-like basket of anisotropic material woven around the nuclei appeared in a normal manner.

and position (Figs 15, 23, 25 and 26). Thus the requirements suggested in the literature (*e.g.*, 24) as criteria for an adequate keratin-stabilizing system were fulfilled.

The opposite was found after administration of DMBA and MCA (13, 14, 19). The cells did not mature and disintegrate in the manner warranted both for normal mouse epidermis and for the hyperplasia caused by dipole-type tumor promoters. The histochemical stainability of $-SH$ in the cytoplasm of the nucleated cells (Figs 2, 4 and 6) was distinctly stronger than normally, but the stainability of $-S-S-$ was low or nil. In the stratum corneum, the concentration of $-SH$ was similarly high (Fig 6), that of $-S-S-$ nearly zero (Figs 8 and 10).

Two important facts are to be stressed: First, the differences in the stainability of $-S-S-$ between carcinogen and tumor-promoter experiments were *qualitative*—not quantitative only. Second, these features were evident also in experiments with orally administered DMBA and MCA. [Quantitative biochemical determinations (unpublished) will show identical features.] In carcinogen series the anisotropic baskets were severely injured. Accordingly, the keratin-stabilizing system was inadequate.

In typical benign keratinizing skin papillomas provoked by the two stage technique in a resistant mouse strain, characteristic of which is a regression tendency, the distribution of $-SH$ and $-S-S-$ as well as the structure of the keratinization device were nearly normal. This may also help us to understand why these papillomas can regress [*cf.* conclusions in (1, 2, 13, 20, 22, 23)].

Thus there are several types and maturation grades of keratins in the mouse epidermis (*cf.* Figs 8, 9 and 11). It can be concluded that normal keratinization in mouse epidermis is not a simple, passive degenerative event, but an inherited energy requiring differentiation (maturation) process (*cf.* 26, 29), and that carcinogens delay and misguide keratinization. These conclusions are supported by the following additional experimental evidence: (a) In IFE hyperplasia brought about by DMBA and MCA, the life time of the carcinogen injured epidermal cells is highly significantly ($P < 0.001$) longer than that in hyperplasia caused by dipole-type tumor promoters, furthermore, longer than in normal mouse epidermis [(30), *see also* discussions in (1, 21, 22)]. (b) In carcinogen hyperplasia, mitotic figures also occur in the daughter cells (21, 30). (c) the keratin contains cellular remnants, as can be seen under the electron microscope (13), and (d) by reinforcement of energy it has been possible to compel the carcinogen injured epidermal cells to proceed with their differentiation, though this induced keratinization will remain incomplete (31).

Speaking in terms of growth, which consists of both cellular differentiation and cellular proliferation, the following hypothesis can be set up. In the course of experimental skin carcinogenesis in mice, the maturation of epidermal cells is delayed, thus resulting in the lack

of an adequate amount of normally differentiating cells. This defect could activate the epidermis to regenerative proliferation—as does the deficiency in differentiating cells in mechanical traumatization of the epidermis [effect of Deelman (32)], as well as the injury on cellular interfaces brought about by dipole-type tumor promoters (1, 2, 33, 34). In experiments with mechanical traumatization without preceding carcinogen treatment, the equilibrium—i.e., physiological balance between differentiating and undifferentiating cells—is reached when cellular reparation is fulfilled, in experiments with dipole-type tumor promoters, equilibrium occurs when the treatment is interrupted. On the contrary, with continuous exposure of the skin to carcinogen, a balance between normally differentiating and defectly differentiating cells will never be reached, but proliferation continues in a regenerative manner. In experimental skin carcinogenesis with the two-stage technique, variations in the efficacy of the preceding carcinogen treatment gives the cells different potentialities, and these differences give rise to tumors of varying nature as TWORT and ING suggested 33 years ago (35).

SUMMARY

This study concerns the antagonism between the effects of locally and orally administered 9,10-dimethyl-1,2-benzanthracene (DMBA) and 20-methylcholanthrene (MCA), and those of locally applied technical and laboratory-synthesized dipole-type tumor-promoting agents, on histochemically demonstrable protein-bound sulfhydryl ($-SH$), and disulfide ($-S-S-$), as well as on birefringent material in the epidermis of a skin-tumor-resistant mouse strain. The reliability of the staining technique was tested in repeated experiments.

In normal epidermis, the cytoplasm of basal cells contained $-S-S-$ bonds. The nuclei of these cells were surrounded at all sides by a complicated three-dimensional, basket-like cytoskeleton system composed of anisotropic molecular chains on different, intersecting planes. This system could be examined as a whole only by comparing sections cut at right angles to each other. Herxheimer's spirals form that part of this system which appears in sections cut sagittally to the dermoepidermal junction. In sections cut parallel to the cutaneous surface, anisotropy appeared as concentric, perinuclear and paramembraneous rings.

Exposure of the back of these mice to tumor promoters changed neither the localization and stainability of $-SH$ and $-S-S-$ nor the architecture of the anisotropic basket. Thus an adequate keratin-stabilizing system was maintained.

On the contrary, exposure of the skin to DMBA and MCA caused distinct derangements in the localization and reactivity of $-SH$ and $-S-S-$. The stainability of $-SH$ was very strong, but that of $-S-S-$ almost nil both in the nucleated cells and in the stratum corneum. The anisotropic baskets were severely injured. The keratin-stabilizing system was inadequate.

CELL PROLIFERATION IN LYMPHOID TISSUE OF NORMAL AND HYPOPHYSECTOMIZED RATS AFTER IMMUNIZATION WITH FOREIGN PROTEIN

By

PER M. LUNDIN

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Despite extensive research the cellular basis of antibody formation remains imperfectly understood. According to *Fagraeus* (1948), administration of antigenic substances was a powerful stimulus to the formation of immature plasma cells from reticulum cells, especially in the splenic pulp. As the antibody titre curve levelled off these immature plasma cells became transformed into mature plasma cells. In experiments *in vitro* she found that the red pulp contained considerably more antibodies than the lymph follicles and that cultures of thymus, lymph nodes and bone marrow had a low antibody content. Also *Erich* and his collaborators derived the plasma cell from a specific plasmablast and suggest that the possible function of the lymphocytes in inflammation is to act as trephocytes (*Erich & Seifter* 1955). *Coons et al* (1953) showed that fluorescent antigen was fixed in antibody-forming lymph nodes by plasma cells but not by lymphocytes.

Accordingly the relationship between antibody formation and plasma cell proliferation seems to be established. But the part played by the lymphocyte in the immunization reaction is obscure. The histological changes in the lymph nodes—with lymph follicle proliferation and the frequently accompanying lymphocytosis—indicates that the formation of new lymphocytes is enhanced (*Erich & Harris* 1942). Various workers including *Pincus & Johnson* (1954) have found that the P^{32} uptake of the lymph nodes in mice increased following subcutaneous immunization with horse albumin. In young guinea pigs *Gyllenstein et al* (1956) found an augmented P^{32} uptake in the inguinal lymph node after injection of living streptococci in the hind leg. Autoradiography disclosed that the uptake in the germinal centres was high. In the relatively inactive pulp the uptake was higher in areas with a high plasma cell content.

That this increased P^{32} uptake partly is due to DNA renewal—which must be interpreted as a sign of cell proliferation—has been demonstrated by *Fichtelius et al* (1957) who used immunization with per

tussis vaccine and by *Lundin* (1958) who immunized rats with pig serum. Moreover, *Fichtelius et al.* as well as *Lundin* found increased cell proliferation in the thymus. Judging by the degree of DNA renewal per unit time, the thymus is a major producer of lymphocytes, and there is no reason to suppose that the thymus does not participate in such a lymphocytotic response. *Fichtelius et al.* (1961) observed a decrease in antibody titres in S-typhi-H immunized guinea pigs after thymectomy.

The present investigation was designed to study the renewal of DNA and RNA in the thymus, spleen and lymph nodes of rats after immunization with pig serum. Since these organs are all more or less hormone-dependent it was considered interesting to compare with the effect of hypophysectomy upon the immunization reaction (cf. *Lundin* 1958).

MATERIALS AND METHODS

Male albino rats of the Danish strain were used. At the beginning of the experiments they were from 60 to 80 days old and weighed from 170 to 190 g. They were fed a standard diet in the form of a powder consisting of maize flour, wheat gluten, casein and adequate amounts of supplementary vitamins and salts. The intact animals were given tap water to drink, the hypophysectomized ones received 5 per cent glucose solution. Hypophysectomy was performed by the parapharyngeal method under ether anaesthesia. The animals were immunized by pig serum diluted with an equal part of saline in a dose of 0.5 ml intraperitoneally daily for ten days. In the hypophysectomized animals the injections were started the day after operation. At the end of the experiments each animal was given intravenously about 0.25 mC P^{32} and killed by decapitation two hours later. Blood and lymphoid tissues were saved. The amount and specific P^{32} activity of the nucleic acids were determined with a modified Smith-Thannhauser-method described earlier (*Lundin* 1958). The results are given in relative activity, i.e. the specific nucleic acid-P-activity in relation to the H₂A-soluble P activity.

Statistics. The weight of the lymph nodes, thymus and spleen all showed positive regression upon body weight in the intact and hypophysectomized controls. The equations of the regression lines have been calculated and the organ weights of the controls corrected for body weight differences, whereupon the groups were compared by means of the *t* test. Means and standard errors of the means are given in the tables.

RESULTS

The weights of the lymphoid organs and their total content of nucleic acids are given in Tables 1 and 2.

In the intact rats immunization gave rise to a significant increase in lymph node weight ($P < 0.01$). The observed moderate increase in spleen weight is not significant when allowance is made for differences in body weight. The thymus showed a significant weight reduction ($P < 0.01$), which agrees with the slight adrenal hyperplasia.

The hypophysectomized rats exhibited similar changes in principle. The weight increase of the lymph nodes is significant ($P < 0.01$). The difference between the spleen weights is probably significant ($P \approx 0.05$). The thymus weight in the two groups show good agreement.

These organ weight differences are accompanied by matching differ-

TABLE 1

No	Body weight		Weight before treatment		Relative organ weight		Relative organ weight		Relative organ weight	
	Initial	Final	Spleen	Thymus	Lymph node	Adrenals	Spleen	Thymus	Spleen	Adrenals
Intact animals	39	188 ± 3	869 ± 315	967 ± 90	177 ± 51	317 ± 80	463 ± 161	144 ± 48	95 ± 28	169 ± 0.38
Intact + pig serum	9	186 ± 8	1083 ± 796	233 ± 154	241 ± 118	394 ± 188	499 ± 479	120 ± 87	110 ± 64	181 ± 1.28
Hypophysectomized	32	174 ± 4	144 ± 3	540 ± 245	206 ± 118	196 ± 94	147 ± 0.47	379 ± 152	140 ± 77	103 ± 0.34
+ pig serum	8	187 ± 7	159 ± 6	750 ± 553	909 ± 157	237 ± 115	135 ± 0.47	477 ± 388	132 ± 88	151 ± 81

TABLE 2

Concentrations and Total Amount per 100 g Body Weight of RNA and DNA in Lymphoid Organs

No	Spleen				Thymus				Lymph nodes			
	RNA		DNA		RNA		DNA		RNA		DNA	
	I	II	I	II	I	II	I	II	I	II	I	II
Intact animals	39	45 ± 1	908 ± 3	77 ± 13	355 ± 13	57 ± 1	82 ± 3	191 ± 6	44 ± 1	42 ± 1	85 ± 2	80 ± 3
+ pig serum	9	49 ± 2	245 ± 98	76 ± 9	381 ± 41	66 ± 1	72 ± 5	196 ± 6	58 ± 2	63 ± 4	97 ± 3	106 ± 7
Hypophysectomized	31	42 ± 1	158 ± 8	72 ± 3	267 ± 14	55 ± 2	79 ± 5	190 ± 5	48 ± 1	47 ± 4	94 ± 3	131 ± 7
+ pig serum	8	44 ± 2	210 ± 92	78 ± 6	376 ± 58	63 ± 3	80 ± 5	218 ± 11	61 ± 2	92 ± 6	108 ± 4	163 ± 12

Concentration of RNA and DNA in 100 mg wet tissue. (I) 11 mg RNA/100 g final body weight

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Statistics. The weight of the lymph nodes, thymus and spleen all showed positive regression upon body weight in the intact and hypophysectomized controls. The equations of the regression lines have been calculated and the organ weights of the controls corrected for body weight differences, whereupon the groups were compared by means of the t test. Means and standard errors of the means are given in the tables.

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These organ weight differences are accompanied by matching differ-

ences in DNA content (Table 2). Hence the total number of cells in the organs has changed. The alterations in RNA content are proportional to those in DNA content.

The cell proliferation in the organs measured in terms of DNA renewal appears in Table 3. As expected after immunization the lymph nodes in both intact and hypophysectomized rats show a considerable and highly significant increase of cell proliferation ($P < 0.001$ in both groups). The corresponding figures for the spleen of intact rats are also higher but the increase is not significant. The cell proliferation in the thymus increased significantly in the hypophysectomized ($P < 0.02$) but not in the intact rats.

The relative changes in RNA renewal are consistently of the same order as those in DNA renewal.

DISCUSSION

These results thus confirm previous investigations indicating that immunization with foreign proteins is accompanied by accelerated cell proliferation particularly in the lymph nodes. The very considerable increase in DNA renewal observed in the present investigation could theoretically be due to enhanced proliferation of lymphocytes and/or of plasma cells. Considering that the lymphocyte is short lived in the lymph node—either it soon leaves the node or it is destroyed by lymphocytolysis—whilst the plasma cell stays within the lymph node, most of the renewed DNA must surely be attributed to accelerated lymphocyte proliferation. For if production of plasma cells were wholly or mainly responsible they would be accumulated in much larger numbers than the microscopic picture suggests.

The changes in relative RNA production were consistently of the same order as those in DNA synthesis. Most of the RNA renewal is probably associated with production of new protein because of the cytogenesis but some of it would seem to result from the formation of antibody protein. In the spleen the plasma cell proliferation histologically is quite as marked as in the lymph nodes in both intact and hypophysectomized rats. But it showed no increase in RNA renewal in the hypophysectomized and only a slight increase in the intact rats. Hence that proportion of the RNA synthesis which could be due to production of antibody protein should be small and so not possible to measure by the rather coarse technique used here. The fact that the spleen of the hypophysectomized rats exhibited no immunization reaction whatsoever rhymes well with the circumstance that cell proliferation in this organ is highly dependent upon an intact pituitary (Jundin 1958).

Interestingly the thymus showed increased cell proliferation after immunization in the hypophysectomized rats. Accordingly this organ must also participate somehow in the defence mechanism against the foreign protein. The almost complete absence of such proliferation in

TABLE 3
P³² Incorporation in RNA P and DNA P Relative Activity

		Spleen		Thymus		Lymph nodes	
		RNA P	DNA P	RNA P	DNA P	RNA P	DNA P
Intact animals	39	905±0.52	6.43±0.58	12.62±0.27	6.41±0.12	8.65±0.25	2.76±0.10
Intact animals + pig serum	9	1091±1.19	8.28±1.21	12.48±0.27	6.72±0.25	10.42±0.53	4.01±0.31
Hypophysectomized	31	579±0.43	1.67±0.16	9.98±0.47	5.03±0.27	7.94±0.45	2.62±0.18
+ pig serum	8	629±0.69	1.76±0.23	12.21±0.28	6.02±0.27	11.50±0.54	3.99±0.27

of new cells seems predominantly depend upon enhanced lymphocytopoiesis

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the intact rats is surely associated with the stimulation of the adrenals resulting from hyperimmunization, the attendant corticosteroid production inhibiting the formation of new cells (cf *Lundin* 1958)

Hence my findings bear out previous observations that marked lymphocytopoiesis is an essential phase of the immunization reaction (cf *Erich & Harris* 1942) This lymphocytopoietic enhancement is apparently greatest in the lymph nodes but, as manifested by the hypophysectomized rats, occurs in the thymus as well. An increase probably takes place in the spleen, too, but the results are not significant in this study.

Mainly owing to the investigations of *Fagraeus & Coons*, we know that antibody formation and plasma cell proliferation are correlated. But the biological significance of the lymphocytopoiesis remains very obscure. After centrifugation *Harris et al.* (1945) found higher antibody titres in the lymphocyte fraction than in plasma which "seemed to indicate that lymphocytes are instrumental in antibody formation". *Erich & Seifter* (1954) maintained that the function of lymphocytes is to serve as trephocytes. *Yoffey & Courtice* (1956) pointed out that this does not explain why the lymphocytes enter the blood stream: the trephocytic function would best be served by destruction in the lymph nodes. In all probability, such lymphocytolysis actually does take place in the lymph nodes and the spleen.

On the basis of his experience of transfusion of labelled lymphocytes, *Fichtelius* (1957) formulated the theory that lymphocytes, which are formed in the thymus, are transported to the spleen, where they are transformed into plasma cells. The results of the present investigation fit this theory well. However, since the total mass of the lymph nodes is considerably greater than that of the thymus in a normal animal, the quantitative significance of the lymph nodes should be greater.

A new impetus has been given to the old hypothesis that plasma cells can be formed from lymphocytes by *Roberts'* (1960) investigations into antibody formation in transferred lymph node cells. He believed that during the synthesis of antibody the lymphocytes metamorphosed to plasma cells, with intermediate cell types prominent during the most active phase of antibody synthesis. His findings fit in with *Wesslén's* (1952) statement that thoracic duct lymphocytes do not contain antibodies but are capable of producing them *in vitro*.

SUMMARY

The production of new cells in the lymphoid organs in rats after immunization with pig serum has been estimated from P^{32} -incorporation into DNA. Immunization gives rise to significantly accelerated proliferation in the lymph nodes of both hypophysectomized and intact rats, to a significant increase in the thymus of hypophysectomized rats and to an insignificant increase in the spleen of intact rats. This production

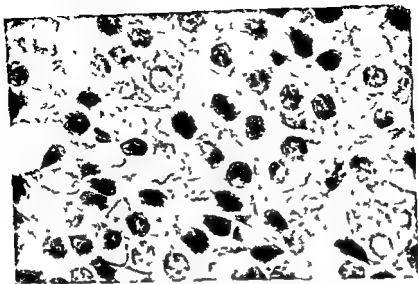


Fig 1

Section of a normal pituitary from an untreated control rat. The alveolar structure is regular with chromophobic, eosinophilic and occasional basophilic cells. Pearse staining. Magnification 1,300 \times (oil immersion).

Specimens intended for solution and stained as sections were fixed for 24 hours and sectioned with glass knives. To examine with an RCA EMU 3 electron microscope. To simplify orientation 1 or 2 μ m.

RESULTS

Among the 10 experimental rats examined so far 6 displayed hypertrophy of the pituitary 2 to 1, months after the commencement of the experiment. After initially gaining weight the rats gradually lost considerable weight. A few exhibited neurological manifestations with pareses and disturbed balance. Two of the 6 enlarged pituitaries from rats killed 8 and 10 months after estrogen implantation were macroscopically definitely tumorous with a softer than normal consistency and large hemorrhages. They grew expansively outside the sella.

Light microscopy. Histological changes had already been induced in the pituitary as early as one month after the start of estrogen exposure. These histological changes were essentially similar in all the non-tumorous pituitaries. The most characteristic features were proliferation of large acidophils, most of which were sparsely granulated and the presence of large apparently ungranulated cells. Both these cell types

LIGHT AND ELECTRON MICROSCOPICAL STUDIES ON THE PITUITARY IN STILBOL-TREATED RATS

By

PIR M. LUNDIN and ULL SCHILIN

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It has long been known that adenomas develop in the anterior pituitary of rats and mice following long-term estrogen treatment (*Cramer & Horning 1936, McEuen, Selye & Collip 1936, Zondek 1936*). Subsequently a long series of papers have discussed the cytological and physiological responses of the anterior pituitary to estrogen treatment, these publications being reviewed in monographs by among others *Clifton & Meyer (1956)* and *Clifton (1959)*.

The estrogen induced tumours have been characterized alternately as chromophobic (*Cramer & Horning 1936, etc.*) and as sparsely granulated acidophilic (*Lacour 1950, Clifton & Meyer 1956, Furlh et al 1956*). Transplantation experiments have shown them to be both mammatropic and somatotropic (*Furlh et al 1956*). Setting in soon after the commencement of estrogen treatment, the reported cytological changes take the form of hypertrophy, division and degranulation of the acidophils, of enlargement and division of the chromophobes, and of concomitant decrease and degranulation of the basophils. The tumours develop after 9 months or longer and consist of enlarged acidophils and enlarged, ungranulated cells resembling those seen shortly after the commencement of estrogen treatment (*Clifton & Meyer 1956*).

The aim of the present investigation was to compare the light microscopical picture and the electron microscopical in order to classify the cell type more reliably and perhaps to provide a basis for an assessment of functional factors.

MATERIALS AND METHODS

The investigation was performed on female Sprague Dawley albino rats initially aged 6 weeks.

Continuous estrogen treatment was assured by implantation of Stilbol tablets each of which contains 5 mg diethylstilboestrol and 15 mg cholesterol. Two such tablets were implanted subcutaneously in the nape of the neck. For this preliminary experiment 10 rats were so treated and 10 others were used as controls.

The rats were killed by decapitation at various times: the first one four weeks after the Stilbol implantation. The pituitaries were removed without undue delay and half the gland was saved for light microscopy and half for electron microscopy.

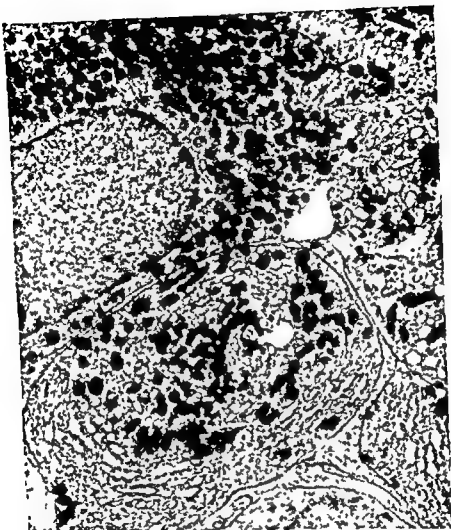


Fig. 3

Normal acidophils in varying stages of secretion. Magnification 14,000 \times

displayed nuclear polymorphism, partially distinct nucleoli, perinuclearly situated negative Golgi apparatuses, and fairly numerous mitoses (Fig. 2). Furthermore was noted a probably diminished proportion of basophils whose cytoplasm often had undergone a measure of degeneration.

In the two tumours the normal pituitary structure was completely eradicated and replaced by a uniform tissue with fairly extensive hemorrhages. The cellular picture was the same as in sections of non-tumorous pituitaries from estrogen-treated rats, with ungranulated

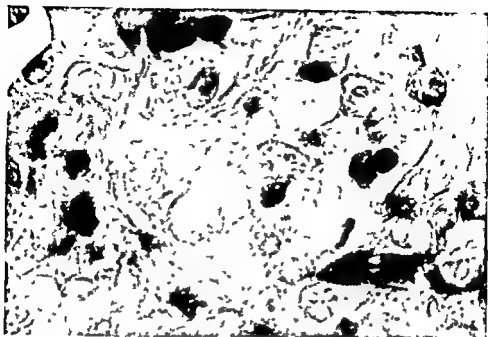


Fig 2

Enlarged and sparsely granulated acidophils and ungranulated cells in section of pituitary from a rat exposed to estrogen for 4 months. Pearse staining. Magnification 1300 \times (oil immersion)

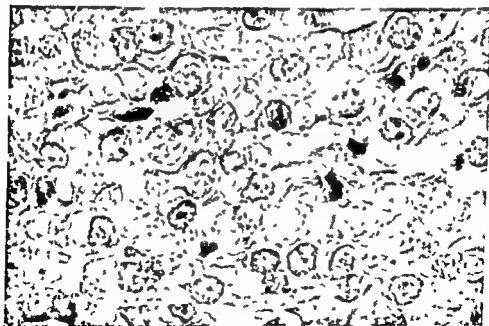


Fig 3

Section of tumorous pituitary from a rat exposed to estrogen for 8 months. Acidophil cell granulation and ungranulated cells are prominent. Pearse staining. Magnification 1300 \times (oil immersion)

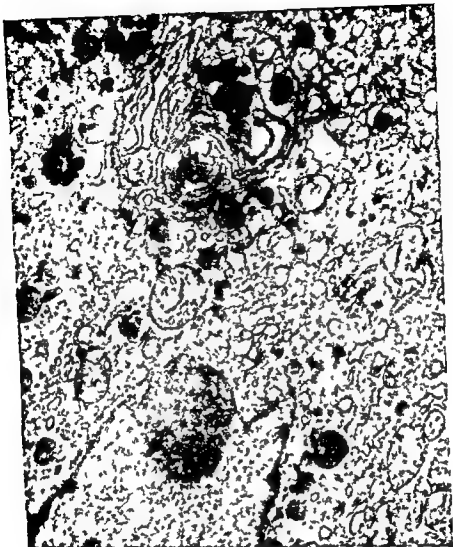


Fig 1

A sparsely granulated cell in a tumour section from a rat exposed to estrogen for 8 months. The secretory activity is marked and the granulation very sparse "Neben kern" Magnification 23 000 \times

few granules. When these cells were compared with their counterparts in similar sections from the untreated controls, it appeared that they were enlarged and had irregular, often bloated nuclei containing one or two prominent nucleoli. In the cytoplasm the Golgi apparatus was markedly enlarged and featured conspicuous vacuoles. The endoplasmic reticulum was strongly developed and occupied the entire cytoplasm in



Fig 5

Proliferating acidophils from a rat exposed to estrogen for 4 months. There is marked secretory activity with strongly developed endoplasmic reticulum and very sparse granulation "Hebenkern" Magnification, 16,000 \times

cells scattered amongst sparsely granulated acidophils, although it seemed more monomorphic and the cells tended to be smaller (Fig 3).

Electron microscopy The electron microscopical sections of non-tumorous pituitaries from estrogen treated rats were completely dominated by cells having a more or less sparse granulation of acidophil type, i.e. moderately osmiophilic granules of regular shape whose magnitude was of the order of 250 to 350 $m\mu$. Some cells only contained



Fig 6

A sparsely granulated cell in a tumor section from a rat exposed to estrogen for 8 months. The secretory activity is marked and the granulation very sparse. Neben kern. Magnification 23,000 X.

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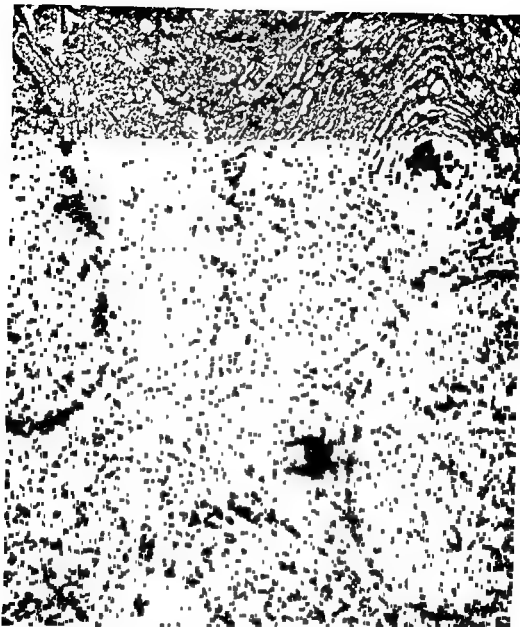


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Under the electron microscope the acidophil cells in Stilbol treated animals consistently showed a pronounced secretory activity and in the tumours no signs whatsoever have so far been encountered of a charging phase with maximal granulation. Hence the weight of evidence

equilibrium. The membrane whorls encountered in the secretory acidophil cells as well as in the tumour cells must be regarded as additional signs of a high secretory activity. Similar formations have previously been reported in estrogen induced tumours by *Haguenau et al* (1955) who named them "Nebenkern" in analogy with the similar light microscopical appearances encountered in the salivary glands and other organs.

Consequently our findings bear out the observations of *Wolfe, Clifton* and other workers suggesting that estrogen stimulates the anterior pituitary. Our electron microscopical observations disclose that the cytological changes affect the acidophil cells and also that estrogen induced pituitary tumours are composed of a single cell type.

SUMMARY

Estrogen treatment of rats gives rise to enlargement of, and the formation of tumours, in the pituitary gland. In the light microscope it has been shown that there is proliferation of acidophils most of which are sparsely granulated and of comparatively large and ungranulated cells. In the electron microscope it became apparent that all the

always are highly secretory. The electron microscopical picture suggests that the acidophil cells in the tumourous pituitaries are at a stage of secretion where hormone production is in equilibrium with hormone excretion.

some cells. Frequently it was disposed in the form of a spiral whose whorls were composed of double membranes—here and there lacking surface granules (Nebenkern)—lying increasingly close together towards the centre. Some secretory granules were generally encountered at the centre of these membranous whorls (Fig. 5).

The cellular picture in sections of the tumours was similar, the cells containing granules of acidophil type although the granulation consistently was very sparse. Apart from less pronounced cell enlargement, the picture conformed on the whole with that in sections of non-tumorous pituitaries. The few secretory granules were situated in the conspicuously developed Golgi zone. In addition there was cytoplasmic dedifferentiation of the type often encountered at electron microscopical examination of tumour cells (Bernhard 1958). Thus the endoplasmic reticulum was split up into short membrane fringes and the RNA particles were found partly scattered or freely aggregated in the cytoplasm. Nevertheless intact portions of the endoplasmic reticulum displayed the same arrangement as in sections of non-tumorous pituitaries (Fig. 6).

Accordingly the tumour cells and the dominating cells in sections of non-tumorous pituitaries from estrogen treated rats must be interpreted as acidophil cells in secretory phase.

DISCUSSION

Numerous previous workers have considered the mechanism of development of the estrogen induced pituitary tumours. Gardner (1948) expressed the view that estrogen caused the tumour to develop by partially inhibiting the hormone production of the anterior pituitary gland. In a later publication (1953) he assumed that estrogen induced tumorous senile lesions of the pituitary cells.

The consensus of opinion seems to be that estrogen stimulates the acidophil activity of the cells. In the estrogen induced tumours, Wolfe (1949) found a large number of hypertrophic, ungranulated cells with enlarged nuclei and Golgi apparatuses, and he went on to propose two means whereby such cells might be formed. Either they could be derived from degranulated chromophil cells that had evacuated their stored hormone and entered a secretory phase so that hormone was produced and discharged at the same rate. Or they could have entered the secretory phase directly from an inactive chromophobic state without first passing through a granulated storing stage.

Clifton & Meyer (1956) believed that the tumours were formed owing to stimulation of the acidophils as well as of "the preacidophil chromophobes" from which the former are derived.

The secretory mechanism in gland cells after various types of stimulation has previously been studied by means of electron microscopy in a number of works especially in pancreas and salivary glands. A strongly developed Golgi apparatus and a sparse granulation with accumula-

tion of granules in the Golgi area has been considered indicative of secretion, while the cells, prior to the secretory stage, are loaded with densely packed secretory granules and a small, compact Golgi apparatus. Similar secretory pictures have been observed in the anterior pituitary by Farquahar & Rinehart (1954) after thyroidectomy and gonadectomy and they hold the opinion that the anterior pituitary cells have a cyclic secretory activity of the same type as has been found in other glands. Similar observations have later been made in pituitaries from mice (Yamada *et al* 1960), cf Fig. 4.

Under the electron microscope the acidophil cells in Stilbol treated animals consistently showed a pronounced secretory activity and in the tumours no signs whatsoever have so far been encountered of a charging phase with maximal granulation. Hence the weight of evidence suggests that the tumour cells are at approximately the same stage of secretion—a fact which seems to verify the aforementioned assumption (Wolfe 1949) that hormone production and hormone excretion are in equilibrium. The membrane whorls encountered in the secretory acidophil cells as well as in the tumour cells must be regarded as additional signs of a high secretory activity. Similar formations have previously been reported in estrogen induced tumours by Hayuenau *et al* (1955) who named them "Nebenkern" in analogy with the similar light microscopical appearances encountered in the salivary glands and other organs.

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HOST FACTORS AND CANCER

Cell Culture Studies

By

J. SAXÉN and K. PENTTINEN

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A great deal has been written on the mechanism of carcinogenesis and especially with reference to the relevant extrinsic factors but relatively little attention has been paid to the host factors in cancer. When one takes into consideration the very many carcinogenic substances, viruses and radiation, it is reasonable to think that during the course of each human life enough stimuli and opportunities should be provided for a cancerous transformation of some cells. That they do not invariably and in all individuals progress to a clinical tumour stage is evidently due to host factors, immunological phenomena and/or the effect of the normal growth controlling mechanism.

Differences in the growth potentials of the cancer cells must be borne in mind but it is probable that differences in host resistance are also important. Everyday the clinician sees that in some persons the tumours grow rapidly and in some slowly—and in some individuals perhaps not at all. The pathologist sees the same on his slides. In some instances the tumours are encapsulated and in others the tissue reaction around tumour cells and cell groups is strong and again at times the host seems to reject the cancer cells as his own normal cells and no reaction whatsoever is seen around them.

During the course of our studies of the normal growth controlling mechanism we have observed permanent differences between fresh normal human sera in their growth controlling effect on cells in cell culture on both normal amniotic cells and cancer cells (12, 14, 16). On repeated feedings with some of the sera cell cultures can even be killed (13).

Since cell growth *in vitro* varies so much in individual fresh human serum, the possibility exists that the growth of cells and cancer cells could also *in vivo* be different in these persons. As the results obtained in preliminary studies seemed to be in agreement with this hypothesis a short report seemed justified.

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cells was observed only at the very end of pregnancy and in some cases of complicated pregnancy. The frequency of sera with strong growth controlling effect and clumping was highest among old patients many of whom had arteriosclerotic heart disease. Serum from cancer patients usually had a good growth promoting capacity, and partial clumping of cells was seldom observed.

DISCUSSION

The series of patients was small, and does not justify the drawing of far reaching conclusions. Most of the sera from cancer patients belonged to the group with good growth promoting capacity. Thus the material from cancer clinics seems to bear out our hypothesis that cell growth *in vitro* in fresh human serum could perhaps also be correlated to the growth of cells *in vivo* in these persons. The small number of cancer patients belonging to the group of partial clumping is noteworthy.

That host defenses against cancer do exist has been suggested many times though studies concerning it are few. The reader is here referred to the recent review by Southam (18).

Of the observations which could be considered as supporting the hypothesis of the great importance of the host factors, the three most important are mentioned here. In heterologous transplantation studies, there is no clear correlation between the growth behaviour of the tumour in its original host (the patient), and its growth in the new host (experimental animals) (7, 19) perhaps indicating that the growth potential of the cancer cell is not the only factor which determines the growth behaviour of a tumour. The host resistance could be even more important.

Cancer cells are often found in the blood of cancer patients, but nevertheless many of these patients are cured by surgery (4), i.e. these persons have obviously been able to control the growth of the disseminated cells.

Homotransplantation studies have demonstrated that more marked and more prolonged growth of implanted cells occurs in cancer patients than in normal persons (17). This could be due to a deficiency in the growth controlling mechanism in the cancer patients.

The nature of the factor active against transplanted lymphomas (8), Landy's natural antibody, which damages mouse tumour cells (9), Bjorklund's cytolytic factor (1), Bolande's properdin studies (2) and Idoroff's studies with serum from schizophrenics (5).

Our earlier observations gave some indication that clumping serum and clumping fractions may contain more low density lipoproteins than

MATERIAL AND METHODS

Use was made of stock cultures of HeLa cells maintained in heat inactivated filtrated human serum pool in Hanks solution. The inoculum size in test tubes (16 × 120 mm) was 20 000 cells per ml. Details of the cultivation system have been earlier published (16). The growth behaviour on the glass surface was recorded after 24 hours stationary incubation in 90 per cent 30 per cent and 10 per cent serum concentration in Hanks solution. In the test series the controls employed always included old non inactivated serum pool, a known serum with strong growth controlling effect (clumping serum), and a known serum in which the cells grow very well in a loose migratory structure (non clumping serum). The sera tested were divided into 5 groups according to the growth behaviour of cells.

No or very weak clumping	1	Excellent growth as in old non heat inactivated serum pool. No clumping of cells even in 90 per cent serum.
	2	Good growth. No clumping in 30 per cent serum but some clumping in 90 per cent serum concentration.
Partial clumping	3	Intermediate group.
Distinct and strong clumping	4	Clumping of cells in 30 per cent serum. No or very few single cells seen between the clumps.
	5	Strong clumping in 30 per cent, partial clumping in 10 per cent, poor or no growth in 90 per cent serum concentration.

The blood samples to be studied were collected in vacutainers, the serum was separated after 24 hours and stored at -70°C , if not used immediately.

The groups of sera studied were: Patients from cancer clinics, old patients (70-90 years), many with arteriosclerotic heart disease, healthy blood donors, children (1-2 years) and pregnant women.

TABLE 1
Growth Behaviour of Cells in Fresh Human Serum from Different Sources

Source of serum	Number of cases	Growth behaviour of cells		
		No or very weak clumping	Partial clumping	Distinct and strong clumping
Blood donors	32	8	16	8
Small children (1-2 years)	9	3	5	1
Patients from cancer clinics	50	33	4	13
Patients of old age (70-90 years)	24	8	1	15
Pregnant women (m II IX)	16	16	0	0
Pregnant women (m X-)	30	15	3	12

RESULTS

The results are presented in Table 1. Healthy blood donors and children formed a very similar group in the respect that most sera belonged to the intermediate group. Serum from pregnant women had usually a very good growth promoting capacity and clumping of

¹ The Table was presented at the VIII Scandinavian Congress of Pathology and Microbiology, June 1-3, Turku, Finland.

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non-clumping serum and its fractions. This again can be in accordance with the results presented here on the growth behaviour of cells in different pathological sera. That low values of β -lipoproteins are often found in cancer patients is reported in literature (10), as is also that an increase in low density lipoproteins is observed in atherosclerosis (15)—where clumping serum seems to be common. That lipoprotein changes do occur in pregnancy is a well known fact. Changes in the recovery rate in cancer cases diagnosed during pregnancy are often reported (6), as is also a high frequency of "in situ" lesions.

The authors are well aware of the fact that correlations such as these are not difficult to find, and that the observed changes may well be concurrent phenomena with no real mutual connection. It should also be pointed out that the observation of good growth promoting effect of serum from cancer patients is not new (11), and that Carrel & Ebeling already in 1922 (3) correlated the growth-inhibiting principles of the serum to the lipid fractions.

Nevertheless, the clumping test we used has given promising results, and more studies along these lines with fresh human serum are needed.

SUMMARY

Great differences exist in the growth behaviour of HeLa cells in fresh individual human serum. Serum from cancer patients and from pregnant women has usually a good growth-promoting capacity, but most sera studied from patients of advanced age, many with arteriosclerotic heart disease, had a strong growth controlling effect. The importance of host factors in the growth of cancer is discussed and stressed.

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RENIN CONTENT OF DIFFERENT PARTS OF THE JUXTAGLOMERULAR APPARATUS

4 *Localization of Renin in the Kidney*

By

JENS BING and JERZY KAZIMIERCZAK

Received 28 vi 61

During the last years the place of renin formation has been studied with indirect methods by *Tobian* (1960) and *Gross* (1960). These studies suggest a high correlation between juxtaglomerular granularity and renin content, which is taken as a sign that renin is formed and located in the part of the vasa afferentia which contain the granules. This opinion, which is in accordance with the conclusions of the many studies by *Goormaghtigh* (1944), *Dunthue & Candon* (1940) and other authors is supported by direct experiments by *Hartroft & Edelman* (1960), in which fluorescent antibodies against a purified, but not pure renin preparation was found to stain the juxtaglomerular granules. Another direct study on the location of renin was performed by *Cook & Pickering* (1959) and *Cook* (1960). They isolated different parts of the nephrons and found the greatest amount of renin in glomeruli with attached fragments of Bowman's capsule and tubular cells.

In previous studies by the authors (*Bing & Wiberg* 1958, *Bing & Kazimierzak* 1959, 1960) the renin content of different parts of the kidney was analysed. In this way it was shown that while renin seemed to be located only in the juxtaglomerular zone, it was not found in measurable amounts, neither in the glomerular capillary loops nor in the part of the glomerular circumference, including the corresponding part of the Bowman's capsule, which is placed opposite to the entrance of the afferent arteriole. Experiments in which the afferent arterioles were isolated as much as possible from the surrounding tubular cells, showed that the tubular cells contained more renin than the afferent vessels, the extracts of the vessels in some cases being without measurable amounts of renin.

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in the isolated vessels. As our previous experiments (1959) have shown that the glomeruli do not contain measurable amounts of renin the content of the vascular preparations (Fig 3) must be due either to renin inside the wall of the afferent arteriole or to "contaminating" tubular cells, which the microdissection failed to remove.

The fact that as much as 40 to 50 per cent may be found in preparations of the as far as possible isolated vessels makes it likely that a lesser or greater part of this is due to location of part of the renin in the wall of the arteriole, and not to insufficiency of the method of removing the tubular cells by the dissection.

As it is unlikely that renin is formed both in the afferent vessels and in the macula densa it was thought of interest to study the renin content as well of nephrons containing the juxtaglomerular granules as of those lacking them. Nephrons belonging to each of these two categories can be obtained from the same kidney, if the kidneys of new born animals are used since they contain an outer row of immature nephrons the vessels of which lack stainable granules as seen by light-microscopy, while the arterioles of the deeper mature nephrons contain the typical granules.

Preliminary experiments, now being continued, have shown that renin is found in the subcapsular zone which contains only immature glomeruli lacking juxtaglomerular granules. This result agrees well with the result of studies of Kaplan & Friedman (1942), who found renin in the mesonephros and metanephros of the hog fetus at a time, when the juxtaglomerular apparatus is undeveloped. It is thus likely that renin is formed in the macula densa and that part of it is deposited in the afferent arteriole which anatomically is closely linked to the macula densa. From there it can easily get access to the lumen of the vessel.

SUMMARY

Previous studies on the localization of renin were continued by determining the renin content of different parts of the juxtaglomerular apparatus in kidneys of cats and rabbits. The following results were obtained:






- (1) From well over 50 to well over 90 per cent of the renin is located in the part of the distal tubule which includes the macula densa.
- (2) A smaller fraction including from less than 10 to less than 50 per cent of the renin is found in the isolated vascular preparations. It is reasonable to believe that in any case where a relatively high percentage of renin is found, this is not only due to "contamination" with tubular cells but also to location of a part of the renin in the wall of the afferent arteriole.
- (3) These results in connection with the results of others and with preliminary experiments on the renin content of different zones of kidneys of new born pigs make it likely that renin is formed in the

was in the macula densa. In order to see if this is the case the renin content of preparations containing a part of the distal tubules including the macula densa, but not the other tubular cells (Fig. 2) was compared with the renin content of preparations containing the total volume of periaarteriolar tubular cells (Fig. 1). In four such experiments (Table 1) it was found, that *the macula densa including parts of the distal convoluted tubules seem to contain the total amount of renin found in the tubules*.

In order to see how much of the total renin is located in the macula densa containing parts of the distal tubules the percentual amount of renin in the isolated vessels (Fig. 3) was determined. In such studies (Table 1) it was found that *in the cat between less than 10 and 40 per cent, and in the rabbit between less than 20 and less than 50 per cent of the renin is found in the isolated vessels*.

TABLE 1

Assays of Renin in Extracts of Different Parts of the Juxtaglomerular Apparatus

Preparation	1	2	3	4	5
					
cat 341	14		50	30	70
cat 349	20		100	30	70
cat 356	15-20		>120 >180	<10	>80
cat 311	12		>120	<10	>90
cat 319		50	>200	<25	>75
cat 323	45 >50-50	40	200 >200	25	75
cat 393	17-14 30	18	45 40	40	60
cat 36	30 60	60			
rabbit 362	35 54	35	>70	<50	>50
rabbit 61	14		>45 77 45	30	70
rabbit 287	20		>100	<20	>80

The renin content of the different preparations are in columns 1 to 3 given by the number of elements needed in the extracts in order to obtain the same pressor response as obtained with one unit of our standard renin preparations. The values in columns 4 and 5 show the approximate amount of renin in 4) the isolated vessels and 5) the macula densa containing parts of the distal tubules given in per cent of the total renin content in preparation 1. With each preparation one or more renin determinations were performed. When more than one preparation were tested the results obtained with each of them are given.

This again means that *in the cat 60 to well over 90 per cent and in the rabbit from well over 50 to well over 80 per cent of the renin was found located in the macula densa containing parts of the distal convoluted tubules, (Table 1 column 5)*.

DISCUSSION

The present studies on the localization of renin in different parts of the juxtaglomerular apparatus have as can be seen in columns 4 and 5 of Table 1 shown that the greater part and in some cases nearly all the renin is found in the macula densa while a smaller part is found

ON THE RENAL RELEASE AND EXCRETION OF ENZYMES IN EXPERIMENTAL NEPHRITIS AND SHOCK

By

LEJVID KEMP and THOMAS LAURSEN

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Release of renin from kidneys has been suggested as the cause of the blood pressure elevation seen in acute glomerulonephritis (Braun-Menendez *et al* 1946, Dexter & Haynes 1944), and as a counteracting factor in severe hemorrhagic shock (Huidobro & Braun-Menendez 1942, Introzzi *et al* 1949, Collins & Hamilton 1944). In two other conditions, acute hypertension after total kidney ischemia and hypertension of the Goldblatt type, where renin liberation has been supposed, release of other renal enzymes into the blood has been demonstrated (Kemp 1959, Kemp 1960, Kemp & Laursen 1960). In experimental shock, Vesell *et al* (1959) has shown release of renal lactic dehydrogenase into the blood. These results support the hypothesis of renin liberation from damaged kidney cells (Kohlstadt & Page 1940, Bing 1945).

The aim of this study was to investigate whether enzymes are liberated from kidneys in animals with acute experimental nephritis and in animals in state of hemorrhagic shock. Determinations of lactic dehydrogenase (LDH), glutamic-oxalacetic-transaminase (GOT), glutamic-pyruvic-transaminase (GPT) and alkaline phosphatase in blood have been carried out and the excretion of LDH in the urine of animals with Masugi-nephritis has been examined.

MATERIAL AND METHODS

A. Nephritis

a. Production of Masugi Nephritis in Rabbits

Ducks were injected intraperitoneally with rabbit kidney homogenate according to the method of Simonsen (1953) which is a slight modification of the original method of Masugi (1933). In later experiments, the kidneys were homogenized in

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macula densa, and that more or less of it is deposited in the afferent arteriole

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GPT after the method of Loursen & Fren Hansen (1958)
 GOT after the method of Loursen & Espersen (1959)
 Protein in urine after the method of Ayer Dayley & Fremon Smith (1931)
 Creatinine after the method of Bonanes & Tausky (1945)
 Blood urea after the method of Conway in the modified form elaborated by
 Brunfeldt & Jacobsen (1948)

RESULTS

A Nephritis

1 Release of Kidney Enzymes

■ Enzyme activity of blood samples As seen in Fig 1, the LDH activity of ear vein blood was high in some cases of acute glomerulonephritis. These high activities were only seen one day during the development of nephritis, (at the time when nephritis was expected—10 days after injection of anti kidney serum the serum LDH activity of ear vein blood was determined every day in up to 5 days). They always occurred before elevation of serum creatinine and just before the onset of proteinuria. It was assumed, but not proved, that the elevated activities were due to release of kidney enzyme, because 1) in 3 samples with high LDH activity the GOT, GPT and alkaline phosphatase were determined and not elevated, and 2) in 3 cases enzyme electrophoresis did not show any sign of elevated liver—LDH—activity. On the other side an attempt to demonstrate a negative renal arteriovenous difference in enzyme activity was unsuccessful (on the contrast positive differences were found in several cases, and in normals too (Table 1 and 2)). A greater activity of the blood after passage through the kidney was never seen. This was possibly due to the sporadic occurrence of cases with high serum LDH activity and the short duration of the elevated activity. The activity was presumably high in only some hours, in two cases two determinations was performed at the same day, and while the first samples showed high activity the activity of the second samples were at the same level as the highest values in the normal group.

TABLE 1
 LDH Activity of Blood Samples Drawn from Aorta and Renal Vein
 Normal Rabbits

Animal no	LDH activity units	
	Aorta	Renal vein
167	53	48
169	70	53
179	102	99
180	214	174
181	124	107
182	75	75
183	69	66
184	132	107
208	165	104

complete Freund's adjuvant and a 10 per cent suspension was produced. This procedure facilitated the immunization procedure. Less kidney tissue and time were required in order to obtain potent nephrotoxic duck serum.

b Criteria for Presence of Nephritis

- 1 Proteinuria ≥ 25 mg%
- 2 Increase in serum creatinine above 1.5 mg%
- 3 Increase in blood urea above 50 mg%

If proteinuria or other of these signs besides proteinuria were found nephritis was said to be present. The urine was examined in all cases, serum creatinine (or blood urea) in almost all cases.

c The Animals

The material consists of 56 white rabbits of both sexes weighing from 2 to 4 kg. Group 1: 10 normals (controls) + 22 animals investigated before the immunization procedure had started.

Group 2: 15 rabbits in which nephritis has not been demonstrated in spite of injection(s) of anti rabbit-kidney duck serum.

Group 3: 31 rabbits with diagnosed nephritis.

d Release of Renal Enzymes

1 Determination of enzyme activity of ear vein blood before and after developed nephritis.

2 Determination of the renal arteriovenous difference in enzyme activity of 9 normal and 21 nephritic rabbits, the operation was performed as described earlier (Kjell 1960).

3 Determination of enzyme activity of kidney perfusates from normals and rabbits with nephritis. Perfusion procedure: after operation when the animal was still alive a cannula was placed in the left renal artery and the kidney perfused with 20 ml 0.9 per cent NaCl at 37° C with a constant perfusion pressure (500 mm H₂O). The perfusate was centrifuged ($\approx 1500 \text{ g}$ for 10 minutes at 20° C) and enzyme activity of the supernatant determined.

4 Determination of IDH activity in the urine before and after developed nephritis.

5 Blood pressure measurements after the method of Grant & Rotzchild (1934) with the modification of Daniel *et al* (1954).

B Shock

a Production and Criteria for Presence of Shock

* intraperitoneally
moral artery until
■ Hg manometer)
ured and when it
no criteria for the

presence of shock were fulfilled in every experiment.

b Release of Renal Enzymes during Shock

1 Determinations of the enzyme activities of blood samples drawn from the femoral or carotic artery during the experiments.

2 Determinations of the renal arteriovenous difference in enzyme activity performed when the animals had been in a state of shock for hours. In these experiments the arterial blood was sometimes drawn from the heart by heart puncture.

Analysis of Heparin Plasma and Urine

IDH after the method of Laurén (1959)

Enzyme electrophoresis after the method of Laurén (1962)

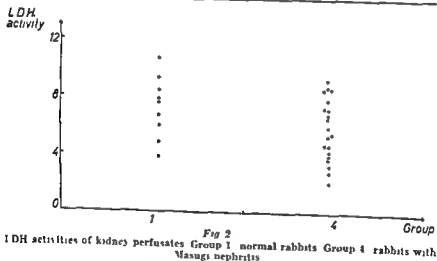
Alkaline phosphatase after the method of Kind & King (1954)

1 We express our gratitude to cand. polyt. Magnusson Statens Seruminstitut for having kindly supplied us with Freund's adjuvant.

TABLE 2

*LDH-Activity of Blood Samples Drawn from Aorta and Renal Vein
Rabbits with Masugi-Nephrit*

Animal no	Aorta	Renal vein
152	31.3	14.8
154	13.4	11.6
155	15.8	15.3
157	24.8	18.5
158	23.1	20.8
159	16.5	15.4
162	20.6	15.5
163	13.3	9.5
164	13.0	14.0
165	9.8	10.5
185	23.0	18.0
187	22.6	8.9
188	12.1	11.9
194	11.7	9.7
196	11.1	8.0
198	26.7	28.2
201	27.9	18.9
204	14.8	13.9
210	11.2	11.2
205	22.7	14.4
192	11.2	7.7



was seen between the degree of proteinuria and enzymuria. In order to investigate whether the concentration of the urine influenced the urine-LDH activity, concentration index for creatinine (urine creatinine/plasma creatinine) was determined in 26 cases and related to the enzyme activity: no correlation was found.

Enzyme-electrophoresis of concentrated urine from rabbits with nephritis showed in all 11 investigated cases LDH-activity belonging to

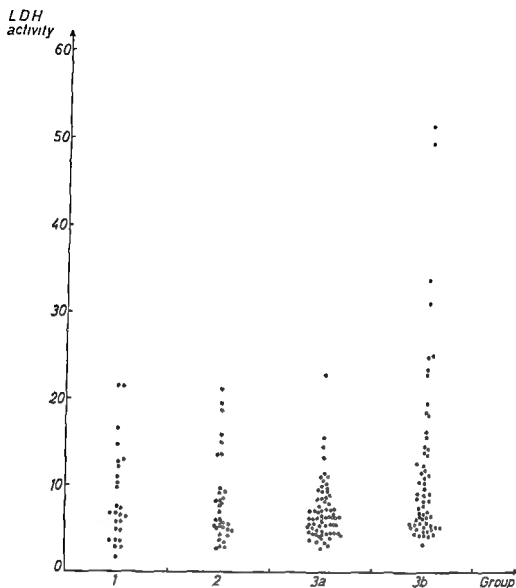


Fig 1

LDH activity of ear-vein blood from rabbits. Group 1 normals. Group 2 rabbits who have received injections of serum from ducks immunized with rabbit kidney extracts without getting nephritis. Group 3 a rabbits who developed nephritis the samples are drawn before or after the week in which the nephritis debuted (group 3 b rabbits who developed nephritis the samples are drawn during the week in which the nephritis appeared).

● = maximal values for each animal ○ = additional determinations

b LDH activity of perfusates No difference in LDH activity of perfusates from normals and rabbits with nephritis was found (Fig 2)

c LDH activity of urine samples A moderate enzymuria was demonstrated in several cases. While only 4 out of 53 urine samples from normals and immunized rabbits without nephritis showed LDH activity greater than 2 units, 30 out of 50 samples from rabbits with nephritis showed LDH activities between 2 and 31 units (Fig 3). No correlation

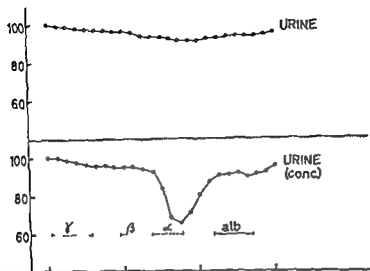


Fig 4

LDH enzyme electrophoresis of urine fr in rabbit with Masugi nephritis. The activity of both unconcentrated and concentrated urine was localized to the alpha globulins. Abscissa: Reading of LDH activity in relation to the distribution of the protein fractions—Ordinate: Galvanometer reading indicating LDH activity (low reading indicates high activity).

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d *Blood pressure measurements*. Blood pressure of 32 normals varied between 50 and 80 mm Hg. In five animals with a very pronounced nephritis a moderate hypertension was developed. In these 5 animals the blood pressure was elevated from 10–20 mm Hg while in 15 other nephritic animals no elevation was seen. High serum LDH activities were not corrected to hypertension.

B Shock

During the shock condition the serum LDH activity became elevated in some cases (Fig 5). This elevation was followed by elevation of serum GOT and GPT (investigated in 2 cases). In three out of 8 cases greater LDH activity of renal vein blood than of aorta blood was found (Table 3).

Enzyme electrophoresis showed LDH activity belonging to several fractions but especially pronounced activity belonging to the gamma globulins was found. Probably this LDH protein is released from the liver. The cats were during the shock in a state of anuria; in one experiment urine production restarted after transfusion. In this case the LDH activity of the urine after shock was 27 units while before the shock it was 11 units.

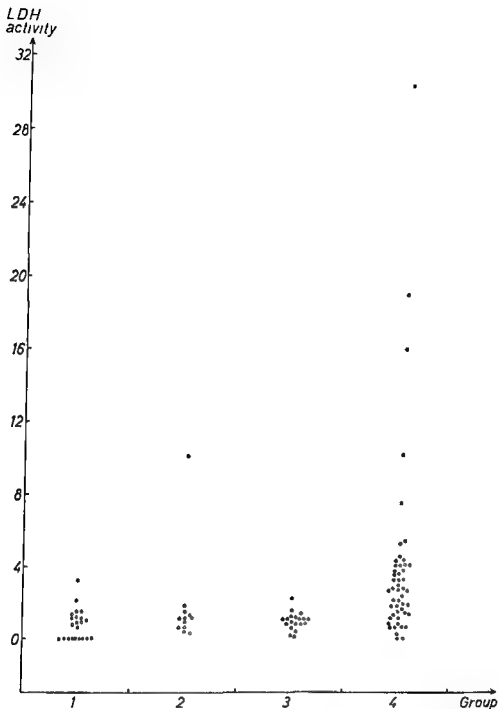


Fig 3

LDH activities of rabbit urine samples. Group 1 normal. Group 2 rabbits who have received injections of serum from ducks immunized with rabbit kidney extracts. These rabbits did not develop nephritis. Group 3 rabbits who developed nephritis in the period before the illness appeared. Group 4 rabbits who developed nephritis

● = maximal values for each animal ○ = additional determinations

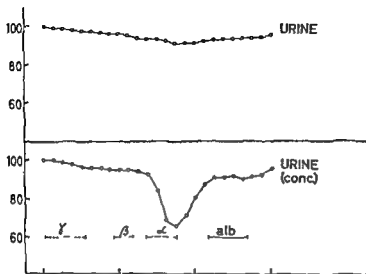


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LDH-enzyme electrophoresis of urine from rabbit with Masugi nephritis. The activity of both unconcentrated and concentrated urine was localized to the alpha globulins. Abscissa: Reading of LDH-activity in relation to the distribution of the protein fractions—Ordinate: Galvanometer reading indicating LDH activity (low reading indicates high activity)

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TABLE 3

LDH-Activity of Arterial Blood (Drawn either from a carotis, aorta or from the Heart) and of Renal Vein Blood from Cats who Have Been in a State of Deep Hemorrhagic Shock for Hours when the Samples Were Drawn

Animal no	LDH activity, units	
	Artery	Renal vein
12	79 (aorta)	86
19	82 (a, carotis)	78
23	142 (aorta)	150
24	29 (aorta)	29
27	35 (cor)	70
28	23 (aorta)	35
30	16 (cor)	133
18	4 (cor)	5

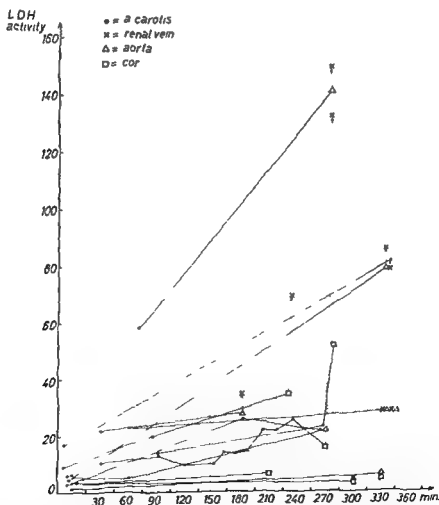


Fig 5

LDH-activity of blood samples from cats during hemorrhagic shock Ordinate: LDH-activity of blood samples drawn from a carotis renal vein, aorta or from the heart after heart puncture Abscissa: Duration of the shock condition in minutes The values of activities from each animal are connected by lines

DISCUSSION

Demonstration of "renin" in blood of patients with acute nephritis has only been suggested in three cases at all, two of which are recorded (without detailed data) in "Renal Hypertension" (Braun-Menendez et al 1946) and one by *Dexter & Haynes* (1944). We agree with the comment of *Pickering* (1955) "Renin would seem to be a possible agent, but the evidence is (again) tantalizingly slight and confused"

In the present work, release of LDH from kidneys of rabbits with acute nephritis was made probable, but a final proof was not obtained. Enzyme electrophoresis of LDH of rabbit kidney tissue does not show a distribution which can be distinguished from the distribution of the enzyme electrophoretic fractions from rabbit heart muscle. Both organs show LDH activity belonging to the α -globulins. In contrast to this, rabbit liver tissue shows a characteristic distribution with high activity in the γ -globulins (*Fritz Hansen, Kemp & Laurien*, in preparation).

In animals with acute nephritis and elevated blood LDH-activity, the enzyme electrophoresis of serum LDH did not indicate the origin of the abnormal serum enzyme. The normal values of serum GOT, GPT and alkaline phosphatases in these cases make a hepatic or cardiac origin improbable. A renal origin seems probable.

This is in agreement with the investigations of *West & Zimmermann* (1958), who found elevated serum LDH activities in many cases of acute kidney disease. On the other hand, as a release of LDH from the kidneys could neither be demonstrated in the present study by the determination of renal arteriovenous differences in enzyme activity, nor in the perfusates of these kidneys, a definite conclusion is not permissible.

The LDH excretion in the urine during acute nephritis was moderate, also when massive proteinuria was present. In all investigated cases, the excreted LDH belonged to the α -globulins. This is in agreement with our earlier investigations of the renal excretion of enzymes after kidney damage: the α LDH from serum is excreted more easily than other isoenzymes (due to a lower molecular weight?). We suppose as *Crockson* (1961) that the LDH in urine from these animals originates from serum- α LDH and not released directly into the urine from damaged kidney cells (*Rosalki & Wilkinson* 1960).

The statement that renin or angiotensin are released from the shock kidney is still questionable. All studies in this field have—in our opinion—given dubious results, due to the lack of a sensitive, specific assay for renin or angiotensin in blood.

As a renin liberation in this condition has not been proved so far, the mechanism of the supposed release is unknown. "Renin" has been demonstrated in the blood few minutes after established hemorrhagic shock (*Hudobro & Braun Menendez* 1942). If renin is released by a cell

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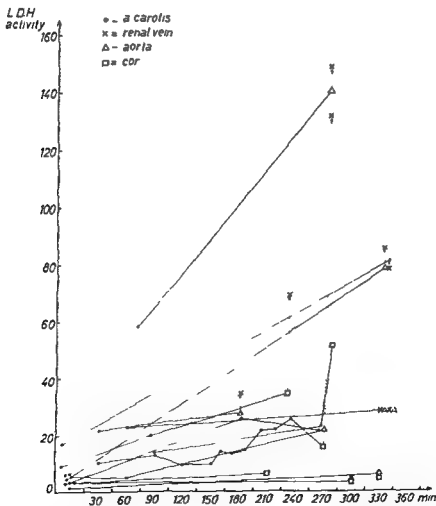


Fig 5

LDH activity of blood samples from cats during hemorrhagic shock. Ordinate: LDH activity of blood samples drawn from a carotis renal vein aorta or from the heart after heart puncture. Abscissa: Duration of the shock condition in minutes. The values of activities from each animal are connected by lines.

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damage as suggested by Kohlstaedt & Page (1940), and Bing (1945), just as other enzymes from damaged tissue (La Due & Wroblewski 1955), renin must leave the kidney more easily than any other enzyme from any organ. On the other hand, demonstration of release of another enzyme (LDH) in conditions where renin-release has been postulated (Kemp 1959, Kemp 1960, Kemp & Laursen 1960 and Vesell *et al* 1959) supports the hypothesis of renin-liberation caused by cell damage.

In hour-lasting deep hemorrhagic shock, a significant renal release of LDH could be demonstrated in 3 out of 8 cases. Enzyme electrophoresis of serum LDH did indicate that a great part of the elevated serum activity was due to release of enzyme from the liver. A parallelism to this has been found in patients with liver diseases and in patients in shock (*Laurson, in preparation*), and agrees with the results of *Vesell et al (1959)*, who found release of LDH from liver, kidney and musculature during this condition.

SUMMARY

The serum and urine lactic-acid-dehydrogenase (LDH) activity of rabbits with Masugi-nephritis, and the serum-LDH activity of cats in acute hour-lasting hemorrhagic shock were investigated. In several cases elevated serum-activities were found in rabbits who developed nephritis. A renal origin of the LDH causing this elevation is suggested (a hepatic or cardiac origin could be excluded), but a final proof has not been obtained. In the urine of animals with Masugi-nephritis moderate amounts of LDH were excreted. This urine activity belonged to the α -globulins, as shown by enzyme electrophoresis.

After hours of hemorrhagic shock a release of renal-LDH into the blood stream could be demonstrated in 3 out of 8 cases

The results are discussed in relation to some of the hypothesis concerning the liberation of renin.

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- [illegible]



Fig. 1

Figs. 1 and 2

Fig. 2

Precipitin pattern of serum from a patient with toxoplasmosis (lower basin) against supernatant of peritoneal exudate from mice infected with the RH strain of *Toxoplasma gondii* (upper basin)

frozen state at -20°C the supernatant gave still the same reaction after 6 months, and at -4°C , preserved with merthiolate, it showed the two precipitation lines after 2 months. Only one line could surely be seen with the portion precipitated at 50 per cent saturation with ammonium sulphate. This gave identity reaction with the line nearest to the serum basin. No activity was found in the precipitate at 100 per cent saturation. During dialysis of the former portion a water insoluble fraction precipitated, but this was inert in the gel precipitation reaction with these two sera, whereas the supernatant was active. Attempt to stain the precipitation lines with Oil red (Gurr's), after washing and drying, failed, indicating that the lipid content of these antigens is low, or that lipid groups are absent.

DISCUSSION

Several authors have pointed to the possible existence of a soluble antigen in connection with *Toxoplasma* infection. Frenkel (1948) (2) and Jirovec & Jira (1961) (6) employed a skin test with supernatant of peritoneal exudate, and found allergic reactions in some patients. Their conclusions were that the concentration of antigen in the supernatant must be low. Observations on the dye test (3, 5) and the fluorescein labelled antibody test (4) show that removal of the supernatant causes some modifications of the results. The authors suggest that this might be caused by a soluble antigen formed by *Toxoplasma gondii* or another modifying effect of the supernatant. Pharmacological studies by Weinman & Klatchko (1950) (8), Woodworth & Weinman (1960) (9) and Debatin (1952) (1) showed that a toxic substance is formed *in vivo* in peritoneal exudate after infection with *Toxoplasma gondii*. Debatin (1) also stated that an antitoxic substance is formed in guinea pigs after inoculation of centrifuged peritoneal fluid which had been freed from viable *Toxoplasma* by freezing and thawing.

SOLUBLE ANTIGENS PRODUCED BY TOXOPLASMA GONDII

By

ASBJORN M. TONJUM

Received 16 vi 61

An extensive literature deals with the serology of *Toxoplasma gondii* particularly the dye test and the toxoplasmin skin test. The complement fixation test, indirect haemagglutination test and a precipitation test with inert particles are also used with crude extracts of *Toxoplasma gondii* as antigens. During the last years the fluorescein labelled antibody test has been employed.

On the other hand it is known of possible soluble antigens produced by this microbe. It therefore seems to be of some interest to give a preliminary report of some studies showing the existence of at least two soluble antigens detected by gel precipitation.

MATERIALS AND METHODS

Gel precipitation. The gel was made from 1.5 per cent Difco agar in 0.05 M veronal buffer of pH 8.2 containing merthiolate 1:10000 and methyl orange 1:5000. A seven basin system on slides was used. The basins were circular and the diameter of each was 3 mm and the distances between them were 3 mm (7). The gel precipitation was carried out at room temperature and the precipitation lines usually were distinct after 1-2 days.

Antisera. Two sera from patients with manifest toxoplasmosis preserved with merthiolate 1:10000. The sera were stored in small portions at -20°C . They showed dye test titers of $\pm 1:3200$.

Antigens. Citrated mouse peritoneal exudate 3-4 days after intraperitoneal infection with the RH strain of *Toxoplasma gondii*. The exudate was centrifuged at 3000 rpm for 30 minutes at 4°C immediately after harvesting and the supernatant constituted the crude material which was used as antigen. It was stored at 20°C until required. The supernatant was treated with ammonium sulphate of 50 per cent and 100 per cent saturation at 4°C . The precipitates were dissolved in 0.05 M veronal buffer of pH 8.2 usually in one third of the original volume and were tested in the gel precipitation reaction. Afterwards the solution was dialysed against distilled water at 4°C .

RESULTS

Untreated supernatant of citrated mouse peritoneal exudate showed two distinct precipitation lines with both sera,—the one nearest to the serum basin was more distinct than the other. The supernatant could be diluted to 1:2 but after greater dilution the reaction failed. Heating of the supernatant to 56°C for 15 minutes removed any visible reaction, after heating to 37°C for 30 minutes the precipitation lines were still seen, but these were now rather feeble. After storing in the

COMPLEMENT FIXING ANTIBODIES IN GUINEA PIGS AFTER INOCULATION OF POLIOVIRUS ANTIGENS

By

JOHN PAPAPANAGIOTOU and HERDIS VON MAGNUS

Received 9/1/61

Guinea pigs respond to inoculation of polio antigens with the formation of neutralizing antibodies, a finding first described by Gard *et al* (1) Hare & Warren (2) demonstrated that also CF antibodies were formed when guinea pigs (GP) were immunized with polio antigens.

In our laboratory GP have been used routinely for potency tests on formalin treated polio vaccines by measuring the formation of neutralizing antibodies in the vaccinated animals after intradermal or subcutaneous injection. It was found of interest to examine whether CF antibodies could also be demonstrated in the vaccinated animals.

MATERIALS AND METHODS

Poliovirus Strains

The virus strains used in our laboratory for routine production of Salk type polio vaccine (3) *i.e.* Brunhilde (Type I), MEF-1 (Type II), and Saukett (Type III) have been employed throughout this study.

Complement Fixing Antigen

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Guinea Pig Sera

Most of the sera studied in the present experiments were derived from animals used for the routine potency tests. The animals were approximately 250 gr.

When the injections of 0.2 ml

1 ml on
or 1/4

¹ Present address: National University of Athens Faculty of Medicine, Department of Microbiology, Athens Greece

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When the intradermal tests (0.2 ml) were finished on day 10, the animals were bled on day 12.

1 Present address: National University of Athens Faculty of Medicine, Department of Microbiology Athens Greece

Complement Fixation Tests

The technique has been described in an earlier publication (5). The complement fixation (CF) tests were performed in glass tubes (Widal tubes) with a total volume of 0.5 per tube. An amount of 0.1 ml each of antigen, serum dilution and complement was allowed to react overnight at $+4^{\circ}\text{C}$. Then 0.2 ml of sensitized 1 per cent sheep erythrocytes was added to each tube followed by 30 minutes of incubation at 37°C . All appropriate controls were included. The test was read after overnight standing at $+4^{\circ}\text{C}$. Fixation greater than 2+ (less than 50 per cent hemolysis) was recorded as positive reaction.

For poliovirus Types I and II, 2-4 units of antigen were employed, while 4 units were used for Type III (5).

Neutralization Tests

Two fold dilutions of serum were prepared in saline and mixed with equal amounts of virus-containing tissue culture fluid representing 50-200 TCD₅₀ per 0.2 ml. The virus-serum mixtures were kept for 1 hour at room temperature and 0.2 ml of each mixture were subsequently inoculated into each of two roller tube cultures of monkey kidney cells (3, 6). The cultures were placed in roller drums at 37°C and read 7 days after inoculation.

Neutralizing antibody titers were expressed as the reciprocal of the highest final dilution of serum protecting 50 per cent of the tubes. The antigenic extinction titer of the vaccines, i.e. the limiting dilution of vaccine causing the formation of antibodies in 50 per cent of the vaccinated animals was calculated by the method of Karber (7).

EXPERIMENTAL

Formation of Neutralizing and Complement Fixing Antibodies after Intradermal and Subcutaneous Inoculation of Trivalent Polio Vaccine

Two vaccines, No. 69 and No. 94, were on 3 different occasions (Experiments A, B and C) inoculated undiluted into guinea pigs, each time one group of 10 animals being inoculated intradermally and another group subcutaneously with vaccine. The immunization schedules are described under "Materials and Methods". All animal sera from one experiment were examined simultaneously.

In Table 1 the results of the neutralization tests and the CF tests have been recorded. It will be seen that both vaccines used undiluted were able to elicit the formation of neutralizing as well as complement fixing antibodies in the guinea pigs.

As regards the CF titers, both vaccines gave in all 3 experiments (A, B and C) the highest values for Type II and the lowest for Type III. Also, in all instances the complement fixing titers were higher in the guinea pigs inoculated intradermally than in the animals vaccinated by the subcutaneous route. This latter finding was least pronounced for Type III.

It should also be noted that the number of responding animals, i.e. animals in each group responding with a complement fixing serum titer $\geq 1/2$, was in all instances highest for the intradermally inoculated animals.

To give an impression of the day to-day variation in the experiments, CF tests on 9 individual guinea pig sera performed on 2 different days

TABLE 1

Formation of Neutralizing and Complement Fixing Antibodies in Guinea Pigs after Intradermal or Subcutaneous Inoculation of Trivalent Polio Vaccines No 69 and No 94

Vaccine no 89										
Inoculation	CP Group no	Neutralizing antibody titer*			CF antibody titer*			Responders CF titer $\geq 1:2$		
		I	II	III	I	II	III	I	II	III
id subc	A	228	100	100	64	160	19	10/10	10/10	8/10
		229	100	78	32	40	12	8/10	8/10	3/10
id subc	B	236	100	204	52	79	16	7/8	7/8	5/8
		237	<31	191	20	52	16	6/10	8/10	6/10
id subc	C	240	129	162	65	171	19	10/10	10/10	8/10
		241	14	93	15	32	12	5/10	8/10	3/10
Total Responders							id subc	27/28 19/30	27/28 24/30	21/28 12/30

Vaccine no 94											
Inoculation	CP Group no	Neutralizing antibody titer*			CF antibody titer*			Responders CF titer $\geq 1:2$			
		I	II	III	I	II	III	I	II	III	
id subc	A	230	234	87	170	92	139	24	10/10	9/10	8/10
		231	129	93	112	19	32	14	8/10	9/10	5/10
id subc	B	238	81	204	123	40	64	15	7/10	8/10	6/10
		239	190	93	145	17	26	12	6/10	5/10	3/10
id subc	C	242	55	347	37	57	187	22	7/9	9/9	7/9
		243	302	302	130	16	26	12	5/10	7/10	2/10
Total Responders							id subc	24/29 19/30	26/29 21/30	21/29 10/30	

* Geometric mean titer expressed as reciprocals of serum dilution

have been recorded in Table 2. It will be seen that the day-to-day variation was very small for all 3 types, and the difference is usually not greater than 2 fold, i.e. one dilution step.

In another experiment the two vaccines No 69 and No 94, were compared with the USA Reference Vaccine No 2 B¹, using the subcutaneous route for inoculation of the guinea pigs.

It will be seen from Table 3 that in the present set up vaccine 94 was found to be a slightly better CF antigen as regards the Type I and Type III components than vaccines 2 B and 69. Neither vaccine 2 B nor vaccine 69 was in the experiment found to elicit Type III CF antibodies in the guinea pigs.

¹ We are grateful to Dr. Roderick Murray, Director of Division of Biological Standards, NIH, Bethesda, USA, for supplying the vaccine.

TABLE 2

CF Antibody Titers Obtained on 9 Guinea Pig Sera on Two Different Days

CP no	Day 1			Day 2		
	I	II	III	I	II	III
9421	4*	4	2	III	4	2
9422	16	32	2	8	16	2
9423	16	32	4	8	32	4
9425	32	16	2	32	16	2
9426	32	32	2	32	64	2
9427	16	32	4	8	32	4
9428	0§	8	0*	8	16	4
9429	16	16	2	8	16	4
9430	0§	16	0§	0§	8	0§
Geometric mean titer	8.6	17.3	2	7.4	15.9	2.5

* Titers are expressed as reciprocals of serum dilution

§ No fixation in dilution 1:2

TABLE 3

*Antigenic Potency of 3 Polio Vaccines as Determined by the Formation of Neutralizing and CF Antibodies in Guinea Pigs after Subcutaneous Inoculation**

Cl Cr up no	Vaccine no	Dilution in cut	Neutralizing antibody titer			CF antibody titer			Response Cl titer 1:2		
			I	II	III	I	II	III	I	II	III
234	2 B	1:1 1:10	78	240	123	1:1	5:1	0†	2/10 0.9	7/10 0.9	0/10 0.9
232	69	1:1 1:10	87	138	105	1:5	2:4	0	3/8 1/10	5/8 1/10	0.8 0/10
235	94	1:1 1:10	151	309	135	4:0	4:1	1:3	9/10 0.8	9/10 0.8	6/10 0.8

* For vaccination schedule see Material and Methods

§ Geometric mean of serum titers expressed as reciprocals of serum dilution

† 0 indicates no fixation in serum dilution 1:2

When employed as antigens in CI tests some monovalent vaccines have previously been found to represent slightly—but consistently so—better CI antigens than others (4). A single experiment was therefore made to study whether any difference could be demonstrated in the ability of such vaccines to produce antibodies in guinea pigs. Two Type I and two Type III monovalent vaccines representing good and poor antigens in CI tests were accordingly inoculated subcutaneously into guinea pigs, and the sera from the vaccinated animals were examined for neutralizing and complement fixing antibodies. The results have been recorded in Table 4.

From these data it appears that the good Type I CI antigen (vaccine 577) gave a better CI antibody response in guinea pigs than did

the poor Type I CF antigen (vaccine 571). The difference in titers obtained with the good and poor Type III antigens was not significant. Also the neutralizing antibody titers and the antigenic extinction titers did not differ significantly. It should be stressed that the results presented here are derived from only one experiment.

TABLE 4

Complement Fixing and Neutralizing Antibodies Elicited by Monovalent Polio Vaccines Representing Poor and Good CF Antigens

Vaccine no	Antigen		Response in guinea pigs			CF Group no
	Type	Quality of CF antigen	CF antibody titers*	Neutr antibody titer*	Antig Extinct titer ‡	
571	I	"poor"	9.2	417	3.3	226
577	I	"good"	69.0	91*	3.5	227
557	III	poor	7.4	17‡	4.1	224
558	III	good™	11.3	240	4.0	225

* Each group of animals comprised 10 guinea pigs.

† Geometric mean of serum titers expressed as reciprocals of serum dilution.

‡ Log titer geometric mean.

CF Response in Guinea Pigs to one Inoculation of Live Poliovirus

In the previous experiments all animals were inoculated either twice subcutaneously or 3 times by the intradermal route. In order to explore whether CF antibodies might appear also when the animals were given one inoculation only, a small experiment was performed where the guinea pigs were given one injection with 1 ml of a mixture of equal parts of Type I, II and III live poliovirus (with infectious titers of 10^6 , 10^5 and 10^6 per 0.2 ml respectively, for the 3 types).

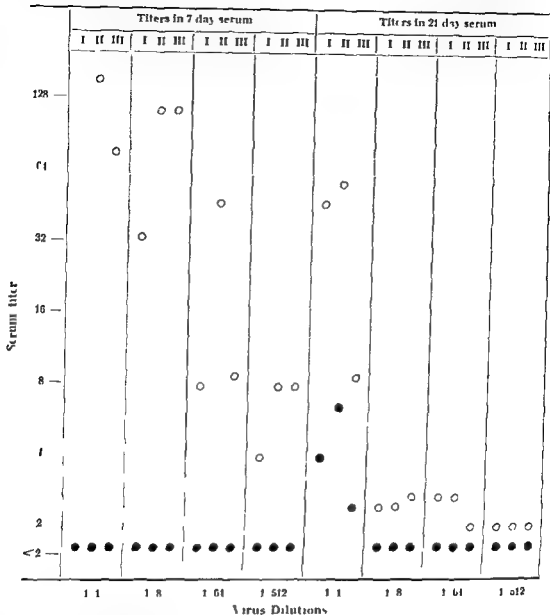
Four groups consisting of 6 animals each were on day 0 inoculated subcutaneously with undiluted and virus dilutions 1:8, 1:64, and 1:512 respectively. Three animals from each group were bled on day 7 and the remaining 3 animals were bled on day 21. The serum samples from the individual animals were examined both in neutralization tests and in CF tests. The titers obtained have been recorded in Figure 1. In the serum collected on day 7 no CF antibodies could be demonstrated while all animals at this time showed neutralizing antibodies for all 3 types of poliovirus. In the 21 day serum the neutralizing antibody titers had dropped considerably while low levels of CF antibodies of all 3 types could now be demonstrated in sera from animals inoculated with undiluted virus.

DISCUSSION

Hare & Warren (2) have previously reported their rather extensive studies on the formation of both complement fixing and neutralizing antibodies in guinea pigs inoculated with live or inactivated polio anti-

Fig 1

Neutralizing and Complement Fixing Antibodies in Guinea Pigs 7 and 21 Days Following Inoculation with 1 ml of Live Poliovirus (a Mixture of Types I, II and III) in Four Dilutions



- Neutralizing antibodies (geometric mean titer in 3 animals)
 ● Complement fixing antibodies (geometric mean titer in 3 animals)

gens. These authors used the intramuscular route for injection of the animals, while in the present study, the injections were given intradermally or subcutaneously. *Hare & Warren* found that after 2 doses of 1 ml of antigen the CF antibodies reached their maximum rather late in the vaccinated animals, *i.e.* 3-4 weeks after the first dose of antigen, an observation which was confirmed in the present study.

With the inoculation schedules employed in the present experiments, higher CF titers were obtained in sera from intradermally injected guinea pigs as compared with the animals inoculated by the subcutaneous route. Also, the number of "responding" animals was higher in the intradermally vaccinated animals. Whether this finding is related to the fact that the intradermal vaccination series consisted of 3 injections while only 2 injections were given to the subcutaneously vaccinated animals, is not known. It should, however, be remembered that the total volume given to each of the latter animals was 2.0 ml, while the total intradermal volume was only 0.6 ml.

As to the practical use of the complement fixation test instead of neutralization tests for testing sera from guinea pigs employed for measuring antigenic potency of formalin treated polio vaccine, no opinion has been formed. Off hand the neutralization test seems more attractive, because correlations between neutralizing antibody formation in children and in guinea pigs have been made. Also, the neutralizing titer values are higher than the corresponding CF titers in the animals' sera.

However, in comparison to neutralization tests the CF test has two advantages. It is quicker to perform, and the CF serum titers are more reproducible (smaller variation) than are neutralization tests. It might therefore well seem worth while to examine the correlation between the CF titers obtained in guinea pigs with a given polio vaccine and the neutralizing serum titers obtained in children vaccinated with the same batch of vaccine.

SUMMARY

After vaccination with formalin inactivated polio vaccine, guinea pigs were found to react with the formation of complement fixing (CF) antibodies as well as with neutralizing antibodies. The CF serum titers showed highest values for Type I and lowest for Type III.

In all experiments the CF titers were higher in guinea pigs vaccinated intradermally than in animals vaccinated by the subcutaneous route.

In one experiment the animals were given one injection of varying amounts of live poliovirus. After one week the neutralizing antibody titer in the animals while the neutralizing antibody titer had fallen off, while CF antibodies were now demonstrable.

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CORRELATION OF THE PLATE DILUTION METHOD TO THE AGAR DIFFUSION METHOD (DISC AND TABLET METHODS) WITH A SPECIAL VIEW TO THE IMPORTANCE OF PRE DIFFUSION

By

V. FROLAND THOMSEN

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At present two groups of methods are available by which the sensitivity of bacteria may be determined *in vitro* viz

- (1) The dilution methods and
- (2) The agar diffusion methods

Originally these methods were used for the determination of contents of antibiotics in sera and tissue fluids but parallel with the rise in demand for these methods have

the methods have become more reliable here a number of substrates is prepared liquid as well as solid containing falling quantities of the drug concerned subsequently the concentration is determined at which full or partial inhibition of growth is obtained. On this basis the degree of sensitivity is estimated on the assumption that corresponding concentrations applied to the site of infection will inhibit growth similarly. The methods however are not widely used in routine testing of sensitivity probably because they are complicated as well as time consuming and also because pure cultures only are applicable if broth dilutions be used subculture is required from the zone of reaction so as to safeguard against contamination.

A more indirect sensitivity test is represented by the agar diffusion method by which advantage is taken of the diffusion into the substrate of antibiotics small samples of the latter being deposited on its surface growth will be inhibited in an area surrounding the deposits the size of the developed inhibition zone being dependent inter alia on the sensitivity of the strain concerned.

The agar diffusion methods are marked by their simplicity of procedure they are inexpensive easily read applicable even in cases of mixed cultures and besides they allow for the determination of several antibiotics on a single plate.

Various modifications are available the filter paper method (here

called the disc method) is the one most commonly used. Filter paper is moistened with adequate solutions of the drug to be tested. When the plate has been seeded with the bacterial culture the discs are placed on the surface and the plate is incubated, either immediately or after a period of pre-diffusion (Jensen *et al* 1948, Lund 1949, Gould & Bowie 1952, Ericsson *et al* 1954, Klein 1953, Grove & Randall 1955, Kirby *et al* 1956/57; Difco Pamphlet No 146, 1958, Morenz 1958). Originally the method represented a simplification of the agar cup method which to day is used but rarely. The method has been described *e.g.* by Bang (1948; Erlanson (1951), and Garrod (1958). The tablet method has gained much ground within recent years (Hoyt & Levine 1947, Lund *et al* 1951, Lund 1955, Braude *et al* 1956, and Schmitt *et al* 1956).

Several other modifications could be mentioned, *e.g.* the "cylinder method", the "drop method", and the "ditch method", it has been discussed widely whether one or the other of these methods is preferable but so far no agreement on this point has been achieved.

The results obtained by the various diffusion methods should be in fair accord with similar results obtainable by the dilution methods. The object of this study is to present investigations in which the correlation of the plate dilution method to the most commonly used diffusion methods has been analyzed. The introduction of a pre-diffusion period is particularly emphasized.

The suitability of the various diffusion methods can be estimated from the accordance between the results obtained by these methods and results obtained in similar studies using the dilution methods.

Previous Studies on the Correlation of the Dilution Method to the Diffusion Method

The correlation between the minimum inhibitory concentrations and the diameters of the inhibition zones may be expressed by a curve shaped as the standard curve of a parabolic curve but reversed (Erlanson 1951) which shall be interpreted to the effect that the ratio of inhibitory concentrations to zone diameters remains constant only in strains exhibiting parallel standard curves. If not parallel the first requirement must be to determine the gradient of this (Bang 1948).

Tung (1951) has compared results obtained by the plate dilution method with those obtained by K & J Jensen's disc method using 100 gram negative rods and 25 gram positive cocci and found a satisfactory correlation. This was also the case in experiments carried out in 1952 by Gould & Bowie who used broth dilutions and a disc method, correlation was absent only in 5 per cent of the tests in which strains were found to be more sensitive by the disc method. Otherwise an almost linear correlation was obtained. Braude & Dockrill (1952) found fair correlation between the tablet method (Diadisc) and the broth dilution method in tests of chloramphenicol, terramycin and penicillin. However correlation was poor in tests of aureomycin in which case the dilution method demonstrated a higher sensitivity. The strains Bondi *et al* (1954) studied the correlation in 125 strains and found deviations only in two of these. Kenney *et al* (1957) emphasized that the resistance frequency would be higher if the disc method were used than if the dilution method were used, however correlation with clinical findings seems to be more satisfactory by the disc method.

Other investigators report more discouraging findings. As early as in 1950 Howe called attention to the fact that the sensitivity of the strains is lower if the disc method be used but the discs used here may have been too weak. Bliss (1951) found

that deviations were so pronounced that she even advised against the use of discs in routine sensitivity tests.

Schwartz & Brown (1954) found results obtained by the disc method almost misleading and recommended the use of a modification of the dilution method in routine work, at about the same time (in 1954) Collins found that deviations were most pronounced in tests with streptomycin in which correlation was found only

on the plates at room temperature for 3 hours after inoculation but before incubation upon which an almost linear correlation between the two methods was ob-

MATERIAL AND METHODS

Laboratory strains or bacterial strains freshly isolated from samples submitted for examination have been used. 132 strains of different genera and degrees of sensitivity are included. The material consists of 54 gram negative rods of the coli klebsiella group, 21 staphylococci, 48 streptococci, 7 pneumococci, and 2 coryneform rods, the streptococci include haemolytic, non haemolytic and faecal streptococci.

A. The Plate Dilution Method

Streptomycin 10 mg per ml of streptomycin solution was used. The plates were inoculated with 0.1 ml of streptomycin solution containing 10 mg per ml of streptomycin. The plates were incubated at 37°C overnight. The reading of results included a count of the number of colonies per plate.

After inoculation the plates were incubated at 37°C overnight.

The reading of results included a count of the number of colonies per plate.

increasing size of the inoculum hazards will also increase of seeding isolated variants of higher resistance thus increasing the MIC but IC50 remains un-influenced since the number of more sensitive bacteria also will increase and hence leave the relative amount of resistant variants constant. The difference between MIC and IC50 does not remain absolutely constant in the different strains but generally it is assumed to equal $\frac{1}{2}$ to 1 dilution stage increasing parallel with increasing inoculum.

The variance (s_d) in these tests is calculated by double determinations at intervals of a few days in 45 strains the experimental conditions of which are kept as uniform as possible. The difference (d) between the two determinations expressed by \log_{10} ranges around zero, the standard deviation of the differences (s_d) is calculated according to the formula

$$s_d = \sqrt{\frac{\sum (d^2)}{2n}} \quad (n=45)$$

s_d is found to equal about 0.05 or 1/6 dilution step. If the hatched value be encircled by a zone of $2 \times s_d$ the correct value with a reliability of 95 per cent will be obtained. Because of the insignificant uncertainty prevailing as regards variations of sensitivity it has been decided to consider values obtained by the dilution method as the ones of highest reliability.

B The Diffusion Methods

Parallel with determinations by the plate dilution method tests using 3 different agar diffusion methods have been carried out:

- (1) the disc method as suggested by *K. A. Jensen (Dragsted et al 1953)*
- (2) the disc method using 5 mm discs and 50 meg of streptomycin (*Kirby et al 1957*)
- (3) the tablet method as suggested by *Lund (1955)* and later followed by comparable determinations in some of the strains using
- (4) the disc method as suggested by *Fricsson et al (1954)* and later
- (5) the disc method applying 6 mm discs and 50 meg of streptomycin together with the introduction of pre diffusion periods of different duration on a few selected strains

re (1)

The disc method as Jensen et al A filter paper disc with a diameter of 20 mm is applied to the non inoculated plate. 0.05 ml of a solution containing 3000 meg of streptomycin per ml is deposited by a pipette. The drug content of the disc is then 150 meg. The plate is left for 30 minutes at room temperature. The paper is removed and the plate is inoculated and incubated.

re (2)

The 5 mm disc method 0.02 ml of a solution containing 2500 meg per ml is applied by micropipette upon which the disc contains 50 meg of streptomycin. After inoculation the discs are placed on the surface and gently pressed on to this and the plate is then incubated.

re (3)

The tablet method as Lund et al The tablets used have diameters of 3 mm and are made of BaSO_4 talcum powder starch and a little staining matter containing 3000 meg of streptomycin. The tablets are applied immediately upon inoculation but before incubation.

These three methods were carried out simultaneously on the same plate to ensure the most uniform experimental conditions.

re (4)

The disc method as Fricsson et al An adequate bacterial suspension is poured over the plate excess being removed by a Pasteur pipette upon which the plate is dried in an incubator at 37°C for one hour. Subsequently the disc is applied and the plate left at room temperature for three hours after which it is re-incubated until the following day. This method can of course not be compared with the above 3 tests on the same plate.

III (5)

Pre diffusion studies 6 mm discs containing 100 mcg of streptomycin and prepared as described under (2) are applied at intervals of 96 48 24 8 2 and 0.5 hours before inoculation of the substrate. Plates are left at 20°C for the above periods when the discs are removed and the plates simultaneously inoculated by identical broth dilutions. Inoculation of the plates is performed by a Pasteur pipette by which 3 drops of the above broth dilution is applied to each plate and spread by a bent glass rod. 15 different bacterial strains were used (cf Table 1).

Substrate 10 per cent blood agar without pepton but with the addition of 1 per cent of glucose was used for all tests. The substrate was poured into 14 cm Petri dishes the thickness of layer averaging 1.0 cm.

RESULTS

Results from tests using the dilution method and the various diffusion methods are illustrated in Figs 1 to 4 in which the IC50 and the diameter of the inhibition zone are shown diagrammatically. The strains

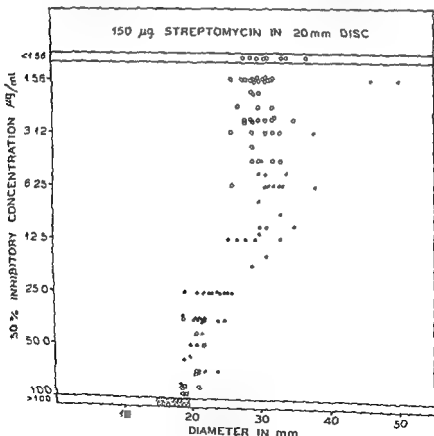


Fig 1

Correlation of the disc method and A Jensen to the plate dilution method

- gram negative rods and staphylococci
- streptococci pneumococci and coryne shaped rods

have been divided into two groups, *viz* group (1) which is marked by an open circle and includes the gram-negative rods and the staphylococci, and group (2) marked by a black dot and including the streptococci, the pneumococci, and the coryneform rods.

Correlation between the IC₅₀ in these strains and the diameter of the inhibition zone for 20 mm discs containing 150 meg of streptomycin is illustrated in Fig 1. Correlation between the two methods is definite but the correlation is different in groups 1 and 2.

In group 1 the difference of inhibition zones is less marked than in group 2, particularly in the sensitive strains. If strains which are inhibited by 6 meg per ml are considered to be sensitive it will be noted that their zones of inhibition all are more than 25 mm wide, but while group 1 is found to range between 25 and 34 mm, group 2 will be found

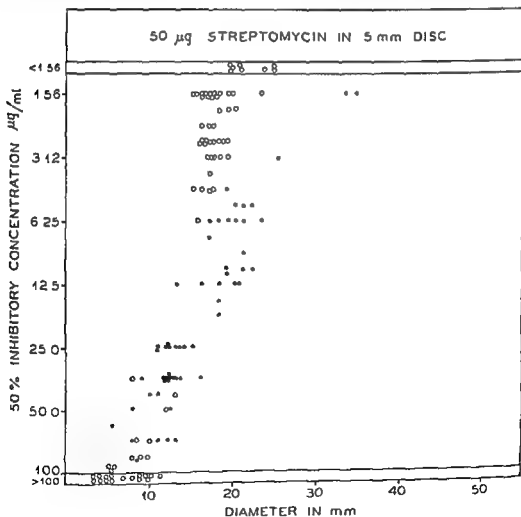


Fig 2

Correlation of the disc method and Kirby et al to the plate dilution method

- gram negative rods and staphylococci
- streptococci pneumococci and coryne shaped rods

to range between 27 and 50 mm. The difference between the two groups is less pronounced in resistant strains and no inhibition zones are obtained in any of the 16 strains in which inhibition fails to occur until concentrations of > 100 mcg per ml are applied.

The results obtained with small 5 mm discs containing 50 mcg of streptomycin are shown in Fig 2. It will be noted that all of the inhibition zones are smaller but that the correlation is almost the same as in the above experiment. Again the zones of sensitive strains in group II are larger than those in group I. Among the 16 strains with $IC_{50} > 100$ mcg per ml were 8 strains i.e. 50 per cent, in which the inhibition zones ranged between 7 and 12 mm.

The results obtained with 9 mm tablets containing 3000 mcg of streptomycin are recorded in Fig 3. The difference between results obtained

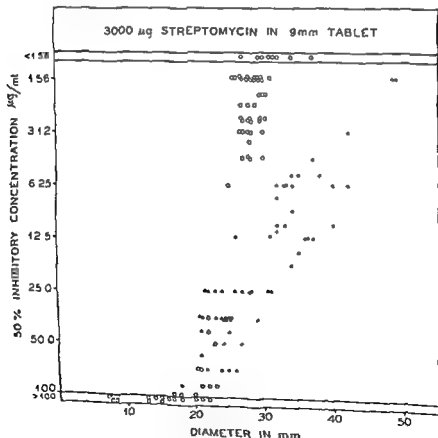


Fig 3
Correlation of the tablet method as in Lund *et al* to the plate dilution method

○ Gram negative rods and staphylococci
● streptococci, pneumococci and coryne shaped rods

in groups 1 and 2 are more pronounced here than above. With a view to the sensitive area group 1 will be found to have separated completely from group 2. Correlation is also more satisfactory in group 2 than in group 1. Among the 16 strains in which the $IC_{50} \geq 100$ mcg per ml were 11 strains in which the inhibition zones ranged between 13 and 22 mm and 5 only in which no inhibition zones developed.

Fig 4 shows results obtained with 5 mm discs containing 50 mcg of streptomycin prepared by the technique suggested by *Ericsson et al*. Only 44 strains were included none of which were fully resistant i.e. $IC_{50} \geq 100$ mcg per ml. Any particular deviation of correlation is compared with the above is not noted. The inhibition zones obtained in group 1 remain inferior to zones obtained in group 2 and here also the difference is most pronounced in the sensitive strains. The regression

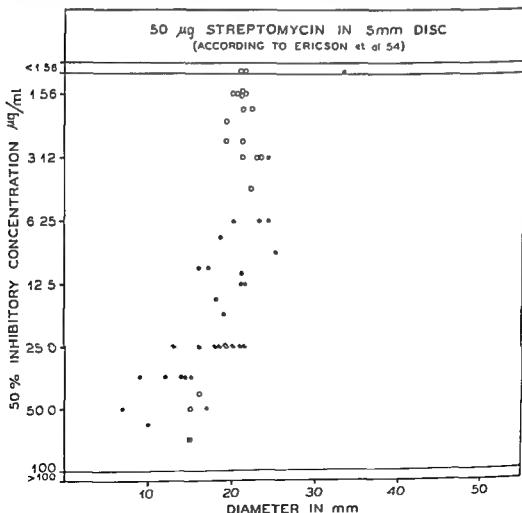


Fig 4

Correlation of the disc method *Ericsson et al* to the plate dilution method

- gram-negative rods and staphylococci
- streptococci, pneumococci and corynebacteria

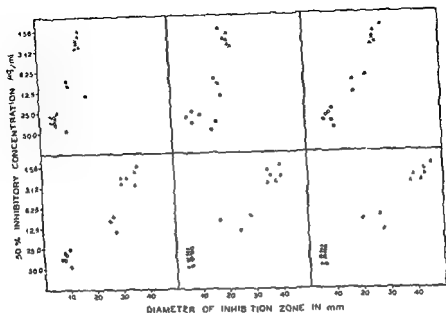


Fig 5

TABLE 1
The Influence of Prediffusion on the Inhibition Zones of Bacteria
of Various Sensitivity (15 Strains)
(Recording Inhibition Zones in mm)

Strains	Species	50% inhibitory con- centration, mcg/ml	Duration of prediffusion in hours					
			0.5	2	8	21	48	96
2	<i>E. coli</i>	15	16	19	29	36	40	16
31	"	23	15	21	26	30	36	43
32	"	18	18	22	26	35	35	43
40	"	26	17	22	27	30	38	42
44	<i>Staph. aur.</i>	25	15	22	26	32	40	40
89	"	28	15	23	25	35	35	38
117	<i>Pneumococ.</i>	13.8	18	19	18	28	25	28
63	<i>Strep. virid.</i>	10.0	11	18	18	26	17	20
131	"	8.3	11	17	20	27	20	27
62	"	35.5	6	8	10	8	<6	<6
130	"	27.8	<6	11	9	8	<6	<6
18	<i>Strep. haem.</i>	29.8	6	6	8	8	<6	<6
69	"	25.0	7	8	10	10	<6	<6
9	<i>Strep. faec.</i>	35.0	<6	17	7	7	<6	<6
12	"	41.0	10	15	11	10	<6	<6

< inhibition zone smaller than the diameter of disc

coefficient of this entity is found to be 1.59 which is very close to the value of 1.64 obtained by *Ericsson et al* thus indicating that the results are compatible

Correlation after Different Periods of Pre-Diffusion

Correlation of the dilution method to the diffusion method after introduction of pre-diffusion periods of varying duration is illustrated in Table 1 and Fig 5

15 strains thus examined are recorded in Table 1, the strains include 4 gram-negative rods and 2 staphylococci which all are considered sensitive, 1 pneumococcus and 2 non-haemolytic streptococci which are considered to be of intermediate sensitivity, and finally 2 haemolytic streptococci, 2 non-haemolytic streptococci, and 2 faecal streptococci which are considered to be relatively resistant. In the 11 sensitive strains the size of the inhibition zone is seen to increase gradually throughout the experiment, in the strains of intermediate sensitivity the increase is gradual during the greater part of the experiment, where increases are rather doubtful in the last part. In the 4 strains which are partially resistant a certain increase is noted initially which, however, within 24 hours is converted into a decrease, and after a period of 48 hours no inhibition zone will remain.

The same results are shown diagrammatically in Fig 5. Here the various groups are marked individually thus making it possible to follow the strains by groups. It will be noted that correlation between the two methods improves parallel with the duration of the pre-diffusion. The cause of this has already been mentioned, viz that whereas zones of sensitive strains will increase the zones of relatively resistant strains will diminish gradually to be finally eliminated. Within 30 minutes the inhibition zones of sensitive strains will be 15-17 mm, and of partially resistant strains they will be 10 mm, however, after pre-diffusion for 96 hours, the zones of sensitive strains will be 38-40 mm and the partially resistant strains will have inhibition zones of less than 11 mm.

DISCUSSION

In laboratories in which large numbers of tests are performed daily, occasionally on samples of highly complicated and heterogenous flora, it is quite impossible to determine the sensitivity of the individual strains by the dilution method, even if the examination be limited to the smallest adequate number of antibiotics. However, under such routine conditions the agar diffusion methods will prove adequate, provided that results from the tests are sufficiently reliable. These tests are inexpensive and quick, they are easily performed, heterogeneous cultures can be examined, and the sensitivity to 6-8 different antibiotics may be tested on the same plate.

However, it should be emphasized that the inhibition zone as such is

rather unimportant it is of importance only if it can be converted into a certain inhibitory concentration which can be obtained at the site of infection. Hence it is not possible to decide correctly which one of the diffusion methods is the better except by a comparison of these with the dilution method.

The present investigations have shown that not all of the bacterial groups will show uniform correlation of one method to the other. In groups in which bacterial growth is rapid (e.g. gram negative rods and staphylococci) the interrelation is steeper than in groups in which growth is less rapid; the correlation will be found most satisfactory in groups in which the rate of growth is low.

The explanation of this difference is that the distance between two points of fixed and different concentrations is shortest during the earliest stages of the diffusion when variations in the bacterial sensitivity are reflected only to a mild degree in zone sizes. During this period changes in growth conditions and of inoculum will exert the most pronounced influence. At later stages of the diffusion the distance between two fixed concentrations will have increased and hence differences in sensitivities will be more marked.

During protracted periods of pre diffusion

- (1) the difference between sizes of inhibition zones in strains with varied sensitivity will be marked and
- (2) the effect of variations caused by different growth rates and sizes of inoculum will be reduced.

The first item is illustrated by the line of regression between the two methods and as shown in Fig. 5 the gradient will be changing parallel with the duration of the pre diffusion periods. This feature is equally obvious on the basis of the following considerations.

At zero time the concentration in the substrate will also be zero immediately upon application of the disc the concentration in its immediate vicinity will have reached a high level even a level of saturation. After a while a certain diffusion into the substrate will have occurred and concentrations in a small area near the disc will have risen a little. If a certain concentration be imagined this will be seen to travel peripherally but if the central concentration be constant it means that the distance will be increased between two points of fixed concentrations. If the central concentration diminish this feature will be indicative of an elimination of certain concentrations in the plate and also of a steady increase of the distance between two fixed concentrations.

Hence improved differentiation between the various degrees of sensitivity is obtained parallel with the duration of the pre diffusion period; the effect exerted by inoculum lag period and generation time on zones of inhibition decreases simultaneously—always provided that such effect be exerted on the critical time i.e. the interval between

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The same results are shown diagrammatically in Fig 5. Here the various groups are marked individually thus making it possible to follow the strains by groups. It will be noted that correlation between the two methods improves parallel with the duration of the pre-diffusion. The cause of this has already been mentioned, viz. that whereas zones of sensitive strains will increase the zones of relatively resistant strains will diminish gradually to be finally eliminated. Within 30 minutes the inhibition zones of sensitive strains will be 15-17 mm, and of partially resistant strains they will be 10 mm, however, after pre-diffusion for 96 hours, the zones of sensitive strains will be 38-46 mm and the partially resistant strains will have inhibition zones of less than 6 mm.

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The same results are shown diagrammatically in Fig 5. Here the various groups are marked individually thus making it possible to follow the strains by groups. It will be noted that correlation between the two methods improves parallel with the duration of the pre-diffusion. The cause of this has already been mentioned, viz that whereas zones of sensitive strains will increase the zones of relatively resistant strains will diminish gradually to be finally eliminated. Within 30 minutes the inhibition zones of sensitive strains will be 15-17 mm, and of partially resistant strains they will be 10 mm, however, after pre-diffusion for 96 hours, the zones of sensitive strains will be 38-46 mm and the partially resistant strains will have inhibition zones of less than 6 mm.

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In laboratories in which large numbers of tests are performed daily, occasionally on samples of highly complicated and heterogeneous flora it is quite impossible to determine the sensitivity of the individual strains by the dilution method, even if the examination be limited to the smallest adequate number of antibiotics. However under such routine conditions the agar diffusion methods will prove adequate, provided that results from the tests are sufficiently reliable. These tests are inexpensive and quick, they are easily performed, heterogeneous cultures can be examined, and the sensitivity to 6-8 different antibiotics may be tested on the same plate.

However, it should be emphasized that the inhibition zone as such is

Brief periods of pre diffusion either periods of 11 hours as suggested by *Fricsson et al* (1951) or periods of 5 hours as suggested by *Klein* (1953), are insufficient according to the present study, but all things considered, brief periods are preferable to no periods. The reason why a 3 hour period fails to improve the correlation is probably that growth in rapidly growing strains has become accelerated by the process of pre-incubation (*i.e.* incubation of the seeded plate before application of the discs) by which process the effect of the pre diffusion may have been neutralized. If the content be reduced the gradient will be established more rapidly, at least, the inhibition zones will be smaller thereby reducing also the difference of inhibition zones in sensitive and resistant strains. After all, the size of the smallest inhibition zone will never be less than nil.

It has been deemed justifiable to conclude that pre diffusion periods of 24 hours are required in order to obtain a satisfactory correlation between the dilution method and the diffusion method.

The present study is concerned only with streptomycin tests but other antibiotics have been examined similarly and findings from these examinations have been seen to be consistent herewith. On the basis of pre diffusion periods of 20-24 hours a method has been established which may be useful in routine sensitivity tests of bacteria. This method will be detailed in a future paper.

SUMMARY

A series of comparative examinations using different methods of diffusion and plate dilution are discussed. The interrelation of the two groups of methods is found to be contingent on the nature of the bacteria and their rates of growth.

Correlation is found to be most satisfactory if periods of pre diffusion of at least 24 hours be introduced. Such introduction of periods of pre diffusion in routine sensitivity tests of bacteria is recommended.

On the basis of considerations on the process of diffusion the interrelation of pre diffusion and the reliability of the diffusion methods is discussed.

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- Branc ... for determination of
- Branc ... arison with the tube

onset of diffusion and development of inhibition zone—because rates of diffusion in the individual points decreases according to a logarithmic scale (cf *Veslerdal* 1947) and therefore the variations in inhibition zones caused by differences of inoculum, lag-period, and generation time will decrease by a factor which increases parallel with the duration of the pre-diffusion

In case of failure of these assumptions, as seen in cases of penicillinase-producing staphylococci and penicillin, it is impossible to predict conditions on the basis of these considerations

The next question is how to determine the duration of the pre-diffusion period in order to obtain the best result, all things considered

In the present study a period of 24 hours was found most adequate because within this period inhibition zones were still obtainable in the partially resistant strains which after 48 hours would have achieved full resistance, in addition correlation with the dilution method was also found to be satisfactory. The application of discs with higher contents of antibiotics will after 48 hours give inhibition zones even with the partially resistant strains, and simultaneously zones will have increased in strains with a higher sensitivity. The optimal duration of the pre-diffusion period cannot be fixed definitely, it will be contingent on the content in the disc of antibiotics, on the desired zones of inhibition of sensitive strains, and on the temperature at which diffusion occurs

A pre-diffusion period of 24 hours has certain advantages. Plates may be prepared on the first day, the following day the discs may be removed and the plates seeded without causing any delay of the sensitivity testing, the diffusion is left to progress until the plate has become "quiet" and, finally, no particular hazards of contamination are involved

The amount of antibiotics with which the disc is impregnated depends on the duration of the pre-diffusion. If this be fixed at 24 hours it should be the amount which upon such pre-diffusion will develop a central concentration just above the level of interest in clinical procedures, subsequently such disc may serve as experimental medium by which to provide a conversion table for diameters of inhibition zones and inhibitory concentrations since diffusion of the various drugs require different conversion tables. If the inhibition zones are too large they are hardly manageable, either requiring large amounts of substrates or, they may overlap. If large areas of concentrations are to be covered as e.g. in case of penicillin, the test should be subdivided by the use of two discs, one with a low content for the sensitive strains and another with a higher content for the more resistant strains

For other antibiotics the concentrations of interest in clinical work are rather well defined by their toxicity. Here the pre-diffusion gives a more accurate differentiation between sensitive and resistant strains than is possible by routine methods in which growth and diffusion start simultaneously

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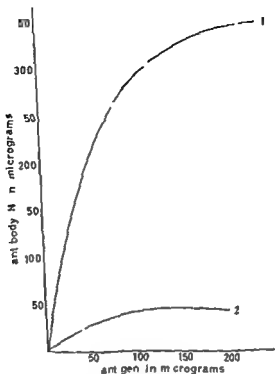


Fig. 1
Precipitation analysis in anti Klebsiella type D immune serum with homologous capsular polysaccharide (curve 1) and the same polysaccharide after oxidation with HIO_4 (curve 2)

of capsular polysaccharide against anti D serum. The amounts of antibody precipitated by different amounts of polysaccharide is shown in Table 2.

TABLE 2
Quantitative Precipitation Analysis in Klebsiella Type D Immune Serum
with Homologous Capsular Polysaccharide

μg antigen added	40	80	100	120	160	200	220	240
μg Ab precipitated	140	260	296	303	327	335	342	346

Table 3 shows the result of inhibition experiments. The antiserum was pretreated with monosaccharide and thereafter precipitated with an amount of polysaccharide.

and quantitative composition as the unoxidized polysaccharide as far as fructose, glucose and mannose are concerned. Uronic acid content

over P_2O_5 in vacuum. Protein impurities were removed from the crude polysaccharide by the method of *Savag* modified by *Heidelberger et al* (2). Finally the polysaccharide was dialysed against frequent changes of distilled water for 3-4 days and reprecipitated.

Hydrolysis and determination of polysaccharide composition. The polysaccharides were hydrolysed in sealed tubes by 3 N H_2SO_4 at $100^\circ C$ for 8 hours. Neutralization of the H_2SO_4 was carried out with $CaCO_3$. The neutralized hydrolysate was lyophilized and redissolved in a small volume of water for chromatography. Different systems were tried and the best separation of the monosaccharides was obtained in pyridine-ethylacetate-water (2:5:5 v/v) using Whatman no. 1 paper.

As standards were used 0.5 per cent solutions of monosaccharides.

Quantitative determination of the constituent monosaccharides was carried out by chromatography in pyridine-ethylacetate-water (2:5:5 v/v) for 24 hours, elution of the spots with water and determination of the individual reducing sugars by the method of *Somogyi* (3). Uronic acid was determined by the method of *Johansson et al* (7). Nitrogen determination was done by the conventional *Kjeldahl* method, phosphorus by *Fiske Subbarow's* method and ash content was determined by incineration in Pt crucibles.

Quantitative serological tests. Quantitative precipitation reactions were carried out as described in an earlier paper from this laboratory by *Henriksen & Friksen* (4).

Inhibition experiments were performed in the way described by *Staub & Tinelli* (5) by incubating antiserum with an excess of monosaccharide for 1 hour at $37^\circ C$ whereafter the precipitation reaction was carried out in the usual way.

Oxidation of the polysaccharide with HIO_4 was done in the cold as described in the above mentioned paper by *Staub & Tinelli*. Oxidation was carried out in the cold for 14 days by 1 N HIO_4 . The acid was removed by dialysis against distilled water and the resulting oxidized polysaccharide was hydrolysed as usual.

Gel precipitation. A few experiments were performed by the method of *Ouchterlony*.

RESULTS

There seemed to be no significant differences between the polysaccharides prepared by the slightly different isolation procedures mentioned. Further polysaccharides prepared from strain MA 1049 and from O 3 seemed to be identical. The identity was established both by qualitative and quantitative determination of constituent monosaccharides and by precipitating the polysaccharides in each other's antisera.

The capsular polysaccharide of *Klebsiella ozaenae* type D seemed to be of the same general type as that of other *Klebsiella* strains investigated in this laboratory by *Eriksen & Henriksen* (6). It contains galactose, glucose, mannose and uronic acid. Table 1 gives the quantitative composition.

TABLE 1
Quantitative Composition in per Cent of the Capsular Polysaccharide

Galactose	Glucose	Mannose	Uronic acid
5.3	22.7	6.3	28.6

Besides these sugars were found 7.2 per cent ash, up to 1.8 per cent N_2 and 0.7 per cent P. Addition of the constituents gives a recovery of about 72 per cent, a rather low value, probably reflecting decomposition during hydrolysis of the polysaccharide.

In Fig. 1 is shown the result of a typical quantitative precipitation test.

saccharide. But since neither qualitative nor quantitative differences between the composition of the native and oxidized polysaccharide were detected it is not known in what manner the oxidation modified the structure of the polysaccharide.

Staub & Tinelli (5) found in their HIO_4 oxidation experiments on *Salmonella* somatic polysaccharide that one of the constituent monosaccharides disappeared on oxidation. The same workers succeeded in inhibiting precipitin reaction between *Salmonella* polysaccharide and homologous immune serum with some of the constituent monosaccharides. This seems to show that both the structure of the *Salmonella* polysaccharide and the nature of the binding between antigen and antibody must be of a different type than that of the corresponding *Klebsiella* system.

SUMMARY

(1) The isolation of the capsular polysaccharide of *Klebsiella ozaenae* type D by three slightly different procedures seemed to give the same product. The polysaccharides of two strains shown by biochemical and serological tests both to be *Klebsiella ozaenae* type D appears to be identical.

(2) The polysaccharide contained 5.3 per cent galactose, 22.7 per cent glucose, 6.3 per cent mannose, 28.6 per cent uronic acid, 7.2 per cent ash, up to 1.8 per cent N, and 0.7 per cent P.

(3) Inhibition experiments attempting to block the precipitin reaction between polysaccharide and homologous antiserum with monosaccharides failed.

Klebsiella ozaenae has ability to precipitate antibody from homologous immune serum.

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was not determined owing to lack of material, but uronic acid was present in the chromatogram. When tested by quantitative precipitin reaction against anti D serum the oxidized polysaccharide precipitated only small amounts of antibody as compared with the intact polysaccharide. This is shown in Fig. 1

TABLE 3

Attempts to Inhibit the Precipitation Reaction between Klebsiella Type D Immune Serum and Homologous Capsular Polysaccharide with Monosaccharides

	$\mu\text{g N}_2$ precipitated by 100 μg polysaccharide
0.5 ml untreated serum	187
0.5 ml serum treated with 40 mg galactose	172
0.5 ml serum treated with 40 mg glucose	174
0.5 ml serum treated with 40 mg mannose	174
0.5 ml serum treated with 40 mg glucuronic acid	179
0.5 ml serum treated with 40 mg galacturonic acid	176

TABLE 4

Quantitative Precipitin Analysis in anti-Klebsiella Type D Serum with Oxidized Polysaccharide as Antigen

μg antigen added	40	80	100	120	160	200
$\mu\text{g N}_2$ precipitated	25	37	42	45	45	43

The amounts of antibody precipitated by different amounts of oxidized polysaccharide are given in Table 4

The gel-precipitation tests showed two lines in the system polysaccharide—anti D serum

DISCUSSION

In spite of the biochemical differences between *Klebsiella ozaenae* type D and *Klebsiella pneumoniae*, the capsular polysaccharide of this type appears to be of a similar chemical constitution as of those types of *Klebsiella pneumoniae* which have been examined until now

Inhibition experiments with the individual monosaccharides found in the capsular polysaccharide, which were carried out in order to gain information about the rôle of these sugars in determining the antigenic specificity of the polysaccharide, failed to give such information since none of the sugars caused any inhibition of precipitation. It should be emphasized, however, that the identity of the uronic acid contained in the polysaccharide is unknown, and it may be neither glucuronic nor galacturonic acid. The results obtained with these two substances may therefore not be relevant.

Mild oxidation with HIO_4 apparently destroys or modifies some of the groupings responsible for the antigenic specificity of the poly-

of anti-treponemal factors in unheated guinea pig serum was thought to be one of the possible explanations to this phenomenon

On the basis of these findings unheated human serum was tested for the presence of *Treponema pallidum* immobilizing activity. It was found that unheated human serum alone without any addition of specific serum immobilized the treponemes.

The immobilizing activity of each normal serum tested was demonstrated in the TPI test by comparing the immobilization of the treponemes in unheated and in activated serum respectively. 0.25 ml of a treponema suspension was mixed with 0.25 ml of unheated or inactivated human serum (i.e. 5 times as much human serum as is generally used in the routine TPI test). Sera from 100 blood donors chosen at random were tested. The immobility percentage varied between 100 and 0 with a mean value of 31. Four sera caused total immobilization. With 24 sera the percentage was more than 50 with 44 sera less than 20. The hemolytic activity of each serum was determined. None of the sera showed anticomplementary activity. After inactivation (30 min 56°C) none of these 100 sera showed any activity when tested in the routine TPI test or in the Wassermann reaction.

The immobilization of the treponemes in unheated human serum

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Reference: Hedström, B. Studies on the Complement Activity in the *Treponema pallidum* Immobilization (TPI) Test. A Comparative Study of the Immobilizing and Hemolytic Complement Activity. *Acta path et microbiol scandinav.*, 53: 180, 1961

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THE SUSCEPTIBILITY OF STAPHYLOCOCCAL HOSPITAL STRAINS TO METHYL-PHENYL-ISOXAZOLYL PENICILLIN

By Lars Olof Kallings

Methyl-(3-phenyl-4-isoxazolyl) penicillin¹ is a new orally absorbable synthetic penicillin active against penicillin G-resistant *Staphylococcus aureus* (New Engl J Med 1961). It is produced by the acylation of 6-aminopenicillanic acid with 5-methyl-3-phenyl-4-isoxazolecarboxylic acid. The hydrated sodium salt is here referred to as P 12. This penicillin was found to be markedly resistant to penicillinase (Gourevitch et al 1961).

In the present investigation the sensitivity of staphylococci to penicillin G (Na benzyl pen) dimethox was compared. The cultures were isolated from

Two fold dilution steps of the penicillins in phosphate buffer, pH 6.0 were incorporated in plates of nutrient agar. The plates were incubated for 18 hours at 37°C. The MIC was determined as the first zone of inhibition. The MIC was expressed as the amount of penicillin in 100 µl of the medium. The MIC was expressed as the amount of penicillin in 100 µl of the medium.

The results are summarized in the Table. The growth of 30 strains was not inhibited by 125 or 256 µg/ml penicillin G. Twenty strains were fully sensitive to it. 94 per cent of the strains were inhibited by 2.4 µg methicillin and 97 per cent by

¹ Prostaphlin in the U.S. (Bristol Laboratories) Micropenin in Sweden (Kabi)

BRIEF REPORTS

A METHOD FOR ISOLATION OF *LISTERIA MONOCYTOGENES* FROM FECES AND OTHER HEAVILY CONTAMINATED MATERIALS

By Olav Sandvik and Andreas Skogsholm

Through tests performed over a considerable period of time by Sandvik *et al* (1958) it was possible to isolate *Listeria monocytogenes* from talle (bottom of sheep pen) in a sheeppen where septicemic listeriosis occurred in new born lambs. According to Gray (personal communication 1958) this is the only case known where *Listeria monocytogenes* has been found in feces or talle under natural conditions. Even the most recent literature (Lehnert 1960 Seeliger 1961) apparently does not mention isolation of the organism from such material. In cases with artificial infection however, the organism has proved its ability to survive in feces.

Since several inquiries in connection with this case have been received the method used at the Department of Microbiology and Hygiene Veterinary College of Norway for isolation of *Listeria monocytogenes* from feces talle and other heavily contaminated material will briefly be described.

To the test material equal quantity of physiological saline is added and the mix homogenized in a Waring blender. The homogenized specimen is put aside for 2-3 hours until the supernatant can be taken up by a syringe and used as inoculum. Occasionally it is necessary to centrifuge the specimen at some 1500 rpm for 1 minute in order to get a relatively clear inoculum. This action does not have any apparent influence upon the result.

4-8 mice are inoculated subcutaneously with each suspension. Optimal quantity of inoculum varies from 0.2-0.5 cc. Intraperitoneal inoculations cause a comparatively high rate of nonspecific deaths and this procedure can thus not be recommended.

Nonspecific deaths generally occur after 1-2 days while deaths resulting from *Listeria* septicemia have occurred after 7-12 days. Cultures are made from liver and spleen. In typical cases *Listeria monocytogenes* is obtained in pure culture.

Nonspecific deaths can be kept at a low rate by use of 0.2-0.3 cc inoculum. Possibly the quantity of inoculum may be increased if preliminary tests show little tendency for nonspecific deaths.

The method described has been used for enzootological investigations for some time (Skogsholm unpublished). *Listeria monocytogenes* has been found in a number of samples by this procedure when other commonly used methods for isolation of the organism have proved unsuccessful.

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Received 28.11.61 from the Department of Microbiology and Hygiene Veterinary College of Norway Oslo (Head professor Steinar Hauque)

TRYPONOMA PALIDUM IMMOBILIZING ACTIVITY IN NORMAL UNHEATED HUMAN SERUM—A PRELIMINARY REPORT

By Benqt Hederstedt

In a previous paper (Hederstedt 1961) it was reported that in the TPI test addition of unheated serum from different guinea pigs to inactivated human syphilitic TPI positive serum caused different degrees of immobilization. Unheated guinea pig serum alone did not cause any significant immobilization. Subdetectable amounts

CALCIUM AND VITAMIN B₁₂ BINDING TO PROTEINS IN HUMAN SERUM AND CEREBROSPINAL FLUID

In Vitro Experiments

By

JØRGEN CLAUSEN and TROELS MUNKNER

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in new born rats and mice *Acta path et microbiol scandinav* 54 136-144 1962

... with the ... are homogeneous when tested by other separation methods, but a few of the fractions, in fact are inhomogeneous, mainly gamma, beta 2-A and beta 2 M, which all show immunological homogeneity, but different mobility. For these reasons immuno-electrophoresis seems specially suited for studies on the binding properties of individual proteins and immuno-electrophoresis.

in
8) ... (11), Lous & Halle (13))

This communication is concerned with the *in vitro* binding of vitamin B₁₂ and calcium. The electrophoretic separation was made on sera incubated with Ca⁴⁵ lactate or Co⁵⁷-vitamin B₁₂.

These investigations have been supported by grants from the National Danish League against Multiple Sclerosis

Growth Inhibition of 105 Staph aureus Strains at Various Concentrations of Three Penicillins

MH μg/ml	Number of strains		
	Penicillin G	Dimethoxy phenyl pc	Methylphenyl isoxazolyl pc
16	59	1	1
8	3	4	
4	8	86	
2	4	13	1
1	1	1	39
0.5	3		63
0.25	7		1
All 0.125	20		

0.5-1 μg/ml P 12 One penicillin G resistant strain (phage group I) was inhibited only by 32 μg methicillin or 16 μg/ml P 12

In clinical trials to be reported on later, a strain of *Staph aureus* resistant to all the three penicillins (MIC 32 μg/ml) was encountered

Five *Staph aureus* strains² resistant to P 12 These strains were originally sensitive. After 37 consecutive transfers in a medium of methicillin, 2 strains were resistant to the remaining 3 strains was 32, 64 and 128 μg/ml resp. At the same time P 12 had increased to more than 256 μg/ml for one strain and to 4-64 μg/ml for the others

Conclusions It was found that *Staph aureus* tested were sensitive to 0.5-1 μg/ml of penicillin G, i.e. sensitive to penicillin G, i.e. penicillin G. They were 4-8 times more sensitive to this new drug than to methicillin. Strains resistant to methicillin were also resistant to methylphenylisoxazolyl penicillin

This is the first oral penicillinase-resistant penicillin. As it has hitherto proved to be the most active towards penicillinase producing staphylococci it should offer improved possibilities for treatment of infections due to these bacteria

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Received 18 xii 61 from the National Bacteriological Laboratory Stockholm

² By the courtesy of Dr G. Wallmarl Stockholm

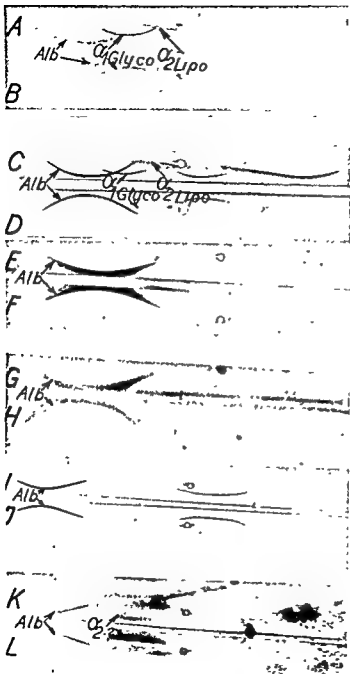


Fig 1

MATERIALS AND METHODS

Human serum, by immuno electrophoresis found to give normal immuno electrophoretic patterns when developed with anti human antiserum obtained from horse from the Pasteur Institute (ASP No 411) was incubated as follows to 10 μ l serum was added 1 μ l B_{12} solution (Philips Roxane) with 0.05 μ Ci (0.5μ Ci = 1/2 microgram B_{12}) to 30 μ l serum was added 22 μ l (0.15 M) lactate (Amersham) solution = 1.9 μ Ci = 0.01 mg B_{12}

Cerebrospinal fluid (CSF) was concentrated to about 7 per cent W/V as already described (Clausen (5)). To 10 μ l concentrated CSF was added 10 μ l Co^{58} vitamin B_{12} solution = 0.5 μ Ci (0.5μ Ci = 3 1/2 microgram vitamin B_{12})

The immuno electrophoresis was performed as described by Scheidegger (18). The auto radiography was performed as described by Clausen & Munkner (6, 7 and 8). The results are given below.

In order to test the results obtained some special criteria were used.

1. Albumin and alpha 1-(S 35) glycoprotein were easy to identify. Albumin corresponded to the largest precipitation line in the anodical area and the alpha 1 (S 35)-glycoprotein to the largest precipitation line in the alpha 1 area crossing the cathodical extremity of the albumin precipitation line.

2. Specific antisera (from rabbits) against albumin, alpha 2 macroglobulin and gammaglobulin were used.

3. The lipoproteins were identified by staining with Oil Red O and Sudan Schwarz (Uriel (22)) and haptoglobins were identified by incubating haemoglobin in serum (Uriel (22)) before the immuno electrophoretic procedure followed by staining for haemoglobin of the immuno electrophoretic preparation.

RESULTS

Vitamin B_{12} Fig. 1 shows the results from the study on *in vitro* binding of vitamin B_{12} to human serum proteins. Three to four lines were developed by the autoradiogram and examined. Among these lines albumin was identified by means of a specific antialbumin-serum and alpha-1-(S 35)-glycoprotein by its mobility and shape. Furthermore an alpha-2-globulin was found to bind vitamin B_{12} . This protein was shown not to be haptoglobin, (see above) the mobility and the shape of the precipitation line suggest that it represents alpha-2-lipo protein. A fourth line of blackening on the autoradiographic film (not seen on the figure) was identified as the intermediate part of the gam-

Fig. 1

In vitro incubation of vitamin B_{12} in normal human serum and concentrated cerebrospinal fluid

- A. Autoradiogram of Co^{58} incubated with human serum. Albumin and alpha 1 (S 35) glycoprotein and alpha 2 lipoprotein have activity.
- B. Autoradiogram of Co^{58} incubated in concentrated cerebrospinal fluid. Albumin and alpha 1 (S 35) glycoprotein have activity.
- C. Immuno electrophoresis of the same as A developed with anti human antiserum obtained from horse.
- D. Immuno electrophoresis of concentrated cerebrospinal fluid developed with anti human antiserum obtained from horse.
- E and F. The same as C and D developed with an anti human antiserum obtained from rabbits.
- G and H. The same as E and F developed as autoradiogram.
- I and J. Immuno electrophoresis of concentrated cerebrospinal fluid developed with anti human antiserum obtained from horse.
- K and L. The same as I and J developed as autoradiogram beside the activity found in the albumin bow a high activity was found localized to an alpha 2 precipitation bow.

This is not the case in paper electrophoresis where also absorption to the paper can disturb the interpretation of the experiments

Vitamin B₁₂ Investigations by means of paper electrophoresis indicate that the vitamin is transported as a complex with alpha 1 and alpha 2 globulins (Pitney & Beard (16) Pitney Marion Beard van Lier (17) Mulgaonkar & Sreenivasan (14) Schull π & Heide (21)) This was found in normal sera by measuring the naturally occurring B₁₂ activity of the fractions as well as of sera to which pure vitamin B₁₂ had been added When great amounts of B₁₂ are added the beta gamma globulin area seems to contain some of the B₁₂-vitamin surplus The previously reported data concerning the B₁₂-binding referred to above have all been influenced by the fact that the electrophoretic areas were inhomogeneous as shown by immuno-electrophoresis The data given in this communication suggest that B₁₂ incubated *in vitro* with serum combines with albumin alpha 1 (S35) glycoprotein and alpha 2 globulin probably identical with alpha 2 lipoprotein and to some extent with the intermediate part of the gammaglobulins

Albumin has already been described as a carrier of many different compounds in blood *e.g.* hormones vitamins and pharmaca (Schull π & Heide (21) Bennhold (234) Antonades (11)) Thus our results from the binding of vitamin B₁₂ to albumin correspond to the previously published data about the binding of vitamins to serum albumin The physiological function of alpha 1 (S35) glycoprotein (Schull π Gollner Heide Schonenberger & Schwick (19) Winter (23)) is unknown Hirschfeld & Sierberg (11) claim that this protein binds the thyroid hormones We are unable to affirm this hypothesis (see our previous results 1960 61) Our finding in serum as well as in CSF indicates that alpha 1 (S35) glycoprotein as well as albumin combine with vitamin B₁₂

The pronounced B₁₂ binding capacity observed in some CSF as compared to serum when studied immuno electrophoretically with the same antihuman antiserum obtained from horses suggests a change of the proteins in CSF Only serum proteins or proteins immunologically identical with serum proteins are visualized in CSF by means of antiserum ASP (No 411) By our procedure the B₁₂-binding CSF serum proteins with alpha mobility must be physico-chemically changed when compared with the corresponding serum proteins without losing the immunological properties of the protein Among the physico-chemical properties which can be changed in a glycoprotein without affecting the immunological properties is the splitting off of neuraminic acid (Schull π & Schwick (20)) by neuraminidase (identical with RDE = receptor destroying enzyme) This splitting off of neuraminic acid causes a decreased mobility of protein after α 1

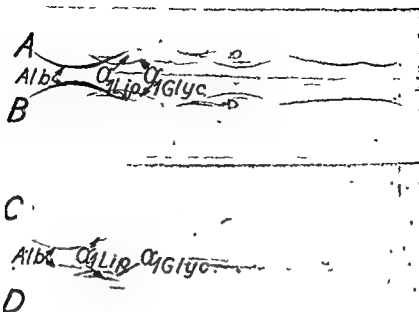


Fig 2

- A and B Immunoelectrophoresis of normal human serum incubated with Ca^{45} developed with anti-human-anti-serum obtained from horse
 C and D The same as A and B developed as autoradiogram. The activity is localized to albumin, alpha-1-lipoprotein and alpha 1-(S 3,5)-glycoprotein

maglobulin. This binding of vitamin B_{12} to the gammaglobulins was variable for reasons which are not evident. Not all sera showed the binding to gammaglobulins with M-mobility, probably because of elution in the washing procedure, which follows the antigen-antibody reaction in order to remove a surplus of antigen and antibody.

Figure 1 also shows the binding of vitamin B_{12} to serum proteins in CSF. Besides albumin the activity is localized to an alpha-1-alpha-2-globulin, which immunologically must be identical (at least in part) with proteins in serum, because antiserum (ASP 411) against proteins in serum was used.

Calcium. Figure 2 shows the *in vitro*-binding of calcium⁴⁵ lactate. Besides the binding to albumin, the main binding occurred to alpha-1-lipoprotein. A weak binding to alpha-1-(S 3,5)-glycoprotein and a diffuse binding to the alpha-2-area, probably to alpha-2-lipoprotein, was demonstrated.

DISCUSSION

Autoradiographs of immuno-electrophoresis of protein solutions with radioactive tracers seem superior to autoradiographs of paper-electrophoresis of the same incubated protein mixture, because the electrophoretic areas obtained in zone-electrophoresis are inhomogeneous, and further because the immuno-electrophoretic procedure involve a step of dialysis, to take away non-reacting antigen and antibody, by which also surplus of added tracer, not protein bound, can be dialyzed away.

the action upon the slowly circulating serum proteins in CSF and probably neuraminidase action on alpha 1 glycoprotein.

The binding to the alpha 2 globulin, probably identical with alpha 2 lipoprotein and to the gammaglobulins with intermediate mobility seems to some extent variable and occurring mainly in serum mixed with excessive quantities of B_{12} .

No binding of vitamin B_{12} to orosomucoid was found which physicochemically is similar to alpha 1 (S 35) glycoprotein (Winkler (23)). But it cannot be excluded that this protein have a binding capacity which cannot be unveiled in immunoelectrophoresis because this protein possesses only weak antigen properties.

Calcium Ca^{++} are firmly bound to carboxyl groups particularly in combination with hydroxyl groups (Laurell (12)). Previous investigations indicate that calcium is bound to albumin (see the survey by Schultze & Heide (21) and Laurell (12) Foster (9)). The binding of calcium to serum albumin increases with increasing pH (Foster (9)). In addition we have found a binding to alpha 1 lipoprotein. This result seems reasonably logical because this protein has threonine as free amino acid with a carboxyl group (Schultze & Heide (21)) and as stated by Laurell (12) the calcium ion is easily bound to hydroxy amino acids as threonine and serine.

The observed binding of calcium to the alpha 1 (S 35) glycoprotein was very slight, a very faint line only being seen in the autoradiogram. No argumentation for the binding can be given since we are ignorant as regards the end groups of this protein.

No calcium binding to orosomucoid was found which has serine as terminal amino acid group (Schultze & Heide (21)). But the antibody titer against orosomucoid is low because the antigenic properties of this protein are very weak (Schultze & Heide (21)) therefore it cannot be excluded that this protein might bind a small amount of calcium. No calcium was found in beta 2 globulin as opposed to the suggestion advocated by Laurell (12). The beta 2 globulin has been shown to have high affinity to zinc and cadmium.

SUMMARY

Autoradiographs following immunoelectrophoresis of serum and cerebrospinal fluids incubated with radioactive vitamin B_{12} and radioactive calcium indicated that vitamin B_{12} is bound to albumin, alpha 1 (S 35) glycoprotein, in alpha 2 globulin probably identical with alpha 2 lipoprotein and to gammaglobulins with intermediate mobility. Occasionally the cerebrospinal fluid seems to possess a high binding capacity of B_{12} to an alpha 1 globulin immunologically identical with a serum protein. Calcium is bound to albumin and alpha 1 lipoprotein and to a smaller extent to alpha 1 (S 35) glycoprotein. The results are discussed on the basis of available data from the literature on proteins.

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TEMPORARY GROWTH OF ROUS SARCOMA (STRAIN MILL HILL) IN NEW-BORN RATS AND MICE¹

By

C G AHLSTROM and N JONSSON

Received 30.11.61

Data in the literature on the possibilities of transferring Rous sarcoma to mammals are contradictory

Rous et al (1912) failed to obtain growth of the chicken sarcoma during post embryonic life in any foreign species. *Vilone* (1928) described 'zig zag' transplantation of Rous sarcoma in fowls and rats but grafts did not survive more than 12 days in rats. *Izujinami et al* (1925-1929) found that Rous sarcoma could survive (but not grow) for some days in an unsusceptible mammal (guinea pigs 11 days rats 6-15 days).

Roskin (1927) has reported growth of the Rous sarcoma in white mice in which the reticulo endothelial system had been blocked with saccharated iron oxide. The growth sustained as long as the blockade persisted but eventually all tumours disappeared.

Shrigley et al (1945) transplanted Rous sarcoma cells to the anterior chamber of the eye of the guinea pig where the transplant initially increased in size and then persisted unchanged for several months. Growth in the guinea pig resulted in changes of the properties of the virus which then produced periosteal tumours in chickens. *Greene* (1951) transplanted the Rous sarcoma to the brains of mice, guinea pigs and rabbits. The incidence of takes was high. Growth was rapid but short lived and invariably terminated in regression of the tumour within 2 weeks of transfer. The tumour could be maintained by consecutive passages from brain to brain. *Ageenko* (1957) also succeeded in transplanting the Rous sarcoma in series to the brains of mice and guinea pigs. The tumours attained maximum size 10-12 days after inoculation after which they showed signs of degeneration and regressed. In mice the tumour was carried through 6 generations, in guinea pigs through 3 and then transferred back to chickens with unchanged biological character. In mice the virus showed no tendency to spread from the brain to other organs. *Alqire et al* (1958) found that grafts from Rous sarcoma grew well in diffusion chambers on mice up to the tenth day, by the thirtieth day all the cells were destroyed.

Rous sarcoma has been transplanted to cortisone treated hamsters and maintained its size for more than 3 weeks (*Kuata et al* 1958-1960). When normal chick embryonic tissue was added to the hamster grown tumours they could be serially transmitted to conditioned hamsters for 3 generations. In non treated hamsters the tumours always regressed within 10 days. *Sigel et al* (1960) found that introduction of minced chicken embryonic tissue treated with Rous sarcoma virus into the brain of conditioned rats was followed by the development of Rous sarcoma of typical appearance. Optimum tumour growth required conditioning by both X radiation and cortisone. The fact that the virus could not be recovered from the rats after injection of Rous virus only points according to these authors to the complete unsusceptibility of this heterologous species to the avian virus.

¹ A preliminary report of this paper was given at the Medicinska Riksstämman in Stockholm on Dec. 20, 1960 and at the Meeting of the Swedish Pathol. Society in Lund on April 15, 1961.

In contrast in recent years various authors have claimed that under certain conditions the Rous virus is pathogenetic for mammals. Zilber *et al* (1957) tried to induce artificial tolerance to Rous virus in rats by inoculating rat-embryos with virus. Subsequent injection of Rous virus into new born rats was followed by a haemorrhagic disease with numerous punctate and diffuse haemorrhages in serous membranes and in the lungs and by the development of axillary and inguinal cysts filled with serous or blood stained fluid. In one case a single injection of the virus in the embryonic period was sufficient to cause the disease. Zilber *et al* (1958) have also reported that Rous virus introduced into new born rabbits can elicit benign fibromatosis. Svet Voldarsky (1957) inoculated rat embryos or new born rats with mixed Rous sarcoma tissue. Six to forty five days after inoculation the rats

development of haemorrhagic cysts and vascular lesions in rats that had been inoculated intra embryonally or during the first days of life with homogenates or tumour cell suspensions of Rous sarcoma tissue whereas intra embryonal and post embryonal injection of virus extract alone had no demonstrable effect. They thought that a

protective effect on the rats. Later administration of anti serum had no effect nor had treatment of the rats with antisera against normal chick tissue (Rychlikova *et al* 1960).

It has been reported that Rous virus can induce not only haemorrhagic disease but also sarcoma in rats. In the experiments of Svet Voldarsky (1958) 3 out of 4 rats inoculated intra embryonally with homogenate of Rous sarcoma developed fatal

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Jain J Iversen (1960) of our laboratory obtained progressively growing metastasizing sarcoma in 2 of 12 rats inoculated at 3 days of age with Rous sarcoma material. It is noted for the lack to

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from Rous sarcoma. The sarcoma material was thoroughly ground with glass powder and suspended 1:10 in Hanks solution with antibiotics. After centrifugation for 10 minutes at 4000 r.p.m. the supernatant was pipetted off and centrifuged again at the same speed for a further 10 minutes. The supernatant was used for subcutaneous inoculation of 10 new born rats and 11 new born mice. 0.20 resp. 0.05 ml was given in each animal. In an additional 11 rats the virus was administered in a suspension of glass powder in an attempt to facilitate the taking of the virus. Cell free material was also given intracerebrally to 13 new born rats.

The inoculated rats and mice were examined every other day for the first few weeks after inoculation, subsequently once a week or once a fortnight. Some of the animals were killed during the first few weeks after inoculation, most of them however not until after 2½-3 months.

The entire material consisted of 286 new born rats and 96 new born mice.

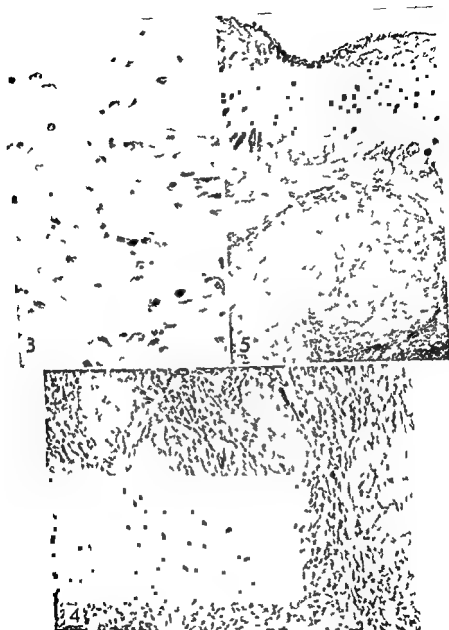
RESULTS

A fairly well defined swelling developed on the back of about one fifth of the new born rats and mice within the first week of subcutaneous inoculation with the suspension of Rous sarcoma. The swelling was discernible at the earliest on the third day after inoculation, but then increased rapidly in size. After a further few days it appeared as a



Fig. 1. Circumscribed oedematous tumour on back of 9 day old rat inoculated with Rous sarcoma with a 24 hours of birth.

Fig. 2. Removal of skin revealed a well defined slimy tumour broadly fixed to underlying tissue in 9 day old rat inoculated at the age of one day with Rous sarcoma.



- Fig 3* 5 x day old tumour built up of elongated cells with oval nuclei arranged irregularly in edematous ground substance
- Fig 4* Eight-day old tumour in rat inoculated at the age of 3 days with Rous sarcoma. The tumor is built up of elongated cells arranged in bundles
- Fig 5* Subcutaneous tumour in 10 day old mouse inoculated at birth with Rous sarcoma

fairly well-defined, elevated, oedematous tumour, which often was broader and thicker anteriorly and tapered off to a narrow tip towards the site of injection into the skin (Fig 1)

The skin over the tumour was not reddened. After having grown for a few days, the tumour regressed. Sometimes the tumour disappeared completely within a few days, but sometimes a small, round, pea sized firm lump persisted for one or 2 weeks. In none of the animals progressive growth was seen for more than at most one week and in none of the animals could any residual swelling be demonstrated for more than 3 weeks after the inoculation. As a rule, regression started when the animals were about 14 days old.

Anatomically the tumour was found to consist of a slimy greyish white mass, which was bordered along its sides by a capsule-like fairly firm tissue (Fig 2). At the regression yellow streaks appeared in the slimy tissue. The small, firm lumps which persisted for some weeks were fibrous and had a yellow centre.

In the early stages the tumours were built up of loosely arranged elongated cells with oblong nuclei and scanty cytoplasm (Fig 3). Some of the cells showed vesicular nuclei with finely dispersed chromatin and distinct nucleoli. Single, sometimes atypical, mitoses were seen. The cells were often branched and arranged in the shape of a loose network in a slimy or oedematous ground substance (Fig 4). No connective tissue fibrils could be discerned. The picture resembled that of the slimy areas in the original tumour in the chicks. In the periphery elongated cells were seen to invade the surrounding muscle bundles. Some days later the lump was delimited by a fairly vascular granulation tissue containing numerous leukocytes and lymphocytes. In the final stage of regression the number of inflammatory cells increased, multi-nucleated giant cells were not infrequently seen around necrotic debris of the tumour.

The anatomical picture was largely the same in rats and in mice (Fig 5).

The course of events was not influenced by cortisone. The tumours appeared at about the same time and did not persist longer than in non-treated controls.

Attempts to transplant the tumour from rats to new-born rats failed. However, inoculation into the breast muscles of 2 chickens with material from two 6-day old rat tumours produced large tumours of Rous-sarcoma appearance within 10 days.

Young rats inoculated intracerebrally with Rous sarcoma material from chicken showed no cerebral symptoms. Nor were any signs of tumour seen on dissection of the brain.

No takes were recorded in animals that had received an intraperitoneal injection of suspended sarcoma cells.

No signs of growth were seen anywhere in any of the new-born rats or mice inoculated with cell free material from Rous sarcoma.

No cysts were obtained in any of the rats and mice inoculated with cellular or cell-free material from the Rous sarcoma

DISCUSSION

There can hardly be any doubt that the oedematous tumours in the new-born rats and mice represented transplants of the Rous sarcoma. This is supported by the short interval between the inoculation and the appearance of the tumours and by their close histological similarity with the slimy growth of Rous sarcoma in chickens. Moreover, material from the tumours transferred back to chickens produced in them the classical picture of Rous sarcoma. The rapid increase in size of the tumours and the mitoses observed in the proliferating cells indicate an active growth and not simply a temporary survival of the transferred sarcoma cells.

It is well known that the capacity to produce antibodies is relatively low in embryos and new-born animals and that this capacity develops only slowly in the young, free-living animal. It is also known that heterologous tumours can grow in new-born rats in which they can be carried in series (Bullock 1915, Gheorghiu 1926, Patti & Moore 1950) but that the tumours are relatively short-lived and disappear within 1 or 2 weeks. In our experiments the Rous sarcoma behaved in the same way as other heterologous tumours: initial growth for a short period followed by rapid, complete regression. The regression occurred at the time when the antibody titre increases in young rats and mice and was presumably due to antibodies against the transferred foreign tissue.

In his attempts to transfer Rous sarcoma from chickens to ducks Duran Reynals (1942) found that the virus, while residing in older, more resistant chickens underwent alterations which increased its capacity to infect the foreign host. Therefore we inoculated the rats not only with Rous sarcoma from chicks but also occasionally with Rous sarcoma from 10-12-month old chickens. The sarcomas grew much slower in these chickens than in chicks, but like the material from the rapidly growing sarcoma in chicks, it failed to produce persistent growth in the rats. Nor did we succeed in facilitating the take of the virus by adding glass-powder to the suspension of Rous sarcoma cells or the filtrate, or by depositing the tumour material in the brain of the rats.

Thus our results are largely the same as those obtained by Rous *et al.*, Milone Shrigley, Greene, Ageenko, Kuwata and Sigel: if there were any difference, it must be that tumours grew less rapidly and gave a lower percentages of takes in our rats and mice.

On the other hand, the results completely deviated from those reported by Zilber *et al.*, Svct-Moldavsky, Schmidt Ruppin, Svoboda and Ising-Iversen. Our animals did not even show a tendency to develop a haemorrhagic disease with bleeding or cysts, and in none of them did

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roughly 14-day intervals. During the 6 months in which the experiments with the Mill Hill strain described in the preceding paper, were in progress the Schmidt-Ruppin sarcoma was kept in glycerol at -40° .

The rats were commercial white rats of the same origin as those used in the preceding investigation. They were inoculated subcutaneously into the back the needle being introduced through the musculature of the right hind leg. The inoculum consisted either of 0.20 ml of finely minced sarcoma tissue suspended 1:5 in Hank's solution with antibiotics or of 0.25 ml of presumably cell free supernatant or of the same amount of a Seitz filtrate.

For the preparation of cell free material the tumour was thoroughly ground with glass powder for 15 minutes and suspended 1:5 in Hank's solution with antibiotics. The suspension was clarified in a MSP "Super mixer" centrifuge for 15 minutes at 4000 r.p.m. the supernatant being cautiously sucked off and transferred to an International Cold Centrifuge where it was centrifuged at 13 800 r.p.m. (10 000 g) for 30 minutes at -3° . The supernatant was cautiously sucked off and again centrifuged time and speed being the same. The same procedure was repeated once more and the final supernatant was used for the experiments.

In some experiments the supernatant prepared in the way described above was filtered through a Seitz filter.

All of the animals were fed standard commercial pellets with an addition of milk and fresh greens.

RESULTS

1 *Inoculation of Minced Chicken Sarcoma into New-born Rats*

Gross findings. Four litters of newborn rats were inoculated subcutaneously in the back with finely minced material from Schmidt-Ruppin sarcoma, suspended in Hank's solution. A few days after the inoculation several animals showed a more or less circumscribed oedematous swelling on the back similar to the one seen after inoculation with the Mill Hill-strain and described in the preceding paper. The swelling occurred in about the same percentage of rats and it regressed after 4-6 days. Some of the animals died within one week after inoculation or were lost by cannibalism. Thirty-three of the inoculated animals were alive 14 days after inoculation.

While the course of events so far was the same as in the experiments described in the preceding paper, a marked difference occurred in the 4th to 5th week of the experiment at which time rapidly expanding subcutaneous cysts or progressively growing sarcomas appeared in several animals. A number of rats developed both sarcomas and cysts.

As a rule, the first tumours could be palpated 4 weeks after inoculation in one rat even within $3\frac{1}{2}$ weeks, in one rat not until 5 weeks after inoculation. The tumours were localized to the back or right thigh (Figs. 1 and 2). They were firm and rounded and had a smooth surface and were often fixed to the underlying tissue (Fig. 3). Usually the tumours grew rapidly. During the following weeks new tumours often appeared in the back, flanks, or sides of the chest (Fig. 4). On further growth adjacent tumours sometimes coalesced. Within 6 weeks to 2 months the tumours often assumed considerable proportions and frequently crippled the animals severely. For example, a tumour originally localized to the thigh would grow up into the region of the hip and down towards the knee achieving the size of a Tangerine. Tumours of the back

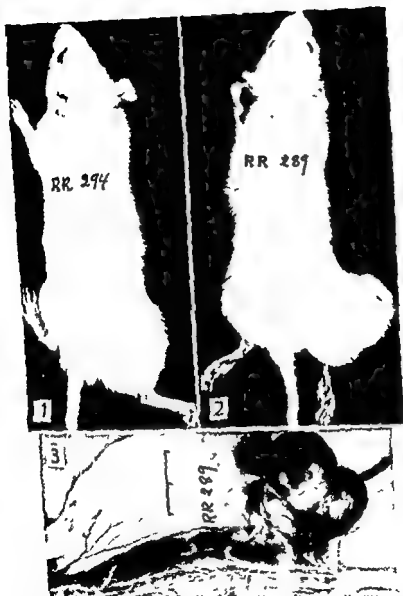


Fig. 1 Rat with a sarcoma in the right thigh 2 months after inoculation, at 4 days of age with minced Schmidt-Ruppin sarcoma

Fig. 2 Rat with a sarcoma in the right thigh 2 months after inoculation at 4 days of age with minced Schmidt-Ruppin sarcoma. Incipient gangrene of the right foot

Fig. 3 The tumour has a nodular surface and is fixed to the thigh muscles



became even larger. Occasionally the growth of the tumour would terminate by complications, making it necessary to kill the animal. Sometimes the growth interfered with the blood supply to the lower leg with gangrene and loss of the foot as a result. A tumour in the region of the hip could invade the pelvis and encircle the rectum; the rats would then show marked coprostasis at the time of death (Fig 5). Tumours in the back were prone to invade the spine and to compress or destroy the spinal cord with consequent paralysis of both hind-legs. Tumours in the cranial part of the back tended to invade the neck and to compress the trachea. The tumours often bulged into the abdomen or into the thoracic cavity.

As a rule, the tumours were firm, but protuberances of some of the large, coarsely nodular tumours were soft. The overlying skin was sometimes adherent to the tumour and then gradually turned a brownish-yellow-colour. In advanced cases the skin over the top of the tumour ulcerated.

In the early stages the cut surface of the tumours was homogenous, greyish-white, moist and shiny but not slimy. The large tumours showed a central yellowish grey, dry, irregular necrosis or multiple small necrotic foci. Occasionally haemorrhages were seen within the tumours. No cystic degeneration was observed.

The sarcomas not only invaded the surrounding tissue but also metastasized. The metastases had the same gross appearance as the primary tumours but were, as a rule, somewhat softer. They were seen most often in the lungs, where they appeared as multiple, small to pea-sized, greyish-white nodules, sometimes elevated over the surface of the lungs (Figs 5 and 6). The contiguous lung tissue was remarkably often haemorrhagic, red-blue or blue-black and rather firm. Metastases were also seen in the mediastinum, where they formed small and large conglomerates of round, greyish-white nodules, not infrequently surrounding the lung hilus and/or the base of the heart. Sometimes the tumour masses in the mediastinum invaded the lungs. Occasionally the thymus was partially replaced by tumour tissue. Sometimes the upper surface of the diaphragm and the inner surface of the thoracic cavity

Fig 4 Rat with two sarcomas, one in the right thigh and one on the right side of the thorax.

Fig 5 The sarcoma in the right thigh has invaded the pelvis and encircled the rectum with consequent coprostasis. Numerous metastases on the upper surface of the diaphragm and on the inner surface of the sternum 2½ months after inoculation with sarcoma.

Fig 6 Numerous metastases died 2½ months after inoculation with sarcoma.

Fig 7 Rat with a sarcoma lymph nodes part of the a part of the a oculation w/ sarcoma cyst in the lower months after in- Schmidt Ruppin





were studded with metastatic growths. Occasionally the pericardium was also covered with metastases, and in one of the animals a secondary growth, the size of a rice-seed, was seen in the myocardium. If the primary tumour were present in the thigh, a chain of lymph node metastases was seen extending from the inguinal region up along the aorta (Fig 7). The lymph nodes involved were more or less enlarged, firm and greyish-white. Sometimes they were surrounded by a narrow haemorrhagic seam. No signs of peritoneal carcinosis were observed not even in cases in which the tumour bulged into the abdominal cavity. In some cases the kidneys were enveloped in tumour masses invading the retroperitoneal space from behind, sometimes the kidneys were partially destroyed but harboured no circumscribed metastases. The animals showed no symptoms suggestive of cerebral metastases, and no such lesions were found in the cases in which the brain was studied *post-mortem*.

Often the cysts appeared first in the groin and or in the region of the axillae and shoulders (Fig 8). In a few of the animals the first cyst developed submentally (Fig 9). The cysts became manifest at about the same time as the tumours and often showed an initially rapid increase in size. Sometimes a bean-sized cyst assumed the size of a hazelnut within a few hours. The cysts were often initially distended to such an extent by their contents that they felt like solid tumours, later, however, they usually became softer, flabby and fluctuant. The content of most of the cysts was blood-stained lending a bluish hue to the overlying skin. A few of the cysts were filled with clear, serous fluid (Fig 9).

Not all of the cysts were localized to the above mentioned areas. Several were seen in the retroperitoneal space, surrounding or displacing one of the kidneys or pressing one of the uterine horns or the colon against the anterior wall of the abdomen (Fig 10). Sometimes symmetric blood-filled cysts were seen, which filled the lower part of the abdomen and were attached to the pelvic wall (Fig 11). On occasion, the cysts assumed enormous proportions and contained as much as 15-20 ml of blood-stained fluid, thus giving the host an almost grotesque appearance. The retroperitoneal cysts or the pelvic cysts often resulted in considerable distension of the abdomen thus simulating ascites. In one animal, which died on the 42nd day after inoculation, the abdomen

Fig 8 Rat with a large haemorrhagic cyst in each axilla and a small haemorrhagic cyst in the left groin 3 months after inoculation at the age of 1 day with minced Schmidt Ruppert sarcoma

Fig 9 Rat with a large cyst on the ventral surface of the neck filled with serous fluid. A small sarcomatous nodule is seen on the left side of the thorax 3 weeks after inoculation with minced Schmidt Ruppert sarcoma

Fig 10 Retroperitoneal haemorrhagic cyst displacing the right kidney upwards 2 months after inoculation with minced Schmidt Ruppert sarcoma

Fig 11 Rat with two big haemorrhagic cysts in the lower part of the abdominal cavity 5 weeks after inoculation with minced Schmidt Ruppert sarcoma



was markedly distended and contained 17 ml of haemorrhagic fluid probably originating from a disrupted haemorrhagic cyst.

The walls of the cysts were thin and were often damaged on dissection. They were sometimes unilocular but often contained thin incomplete septa. When a cyst was damaged during preparation it collapsed to a thin shred. When the cyst collapsed before it had been loosened from contiguous tissue it was often impossible or difficult to identify the wall with certainty. Older cysts had a thicker red brown wall. The surrounding tissue often showed a brownish discoloration.

The cysts were often multiple. Beyond the cysts found by palpation autopsy usually revealed additional cysts. Cysts were never seen in the mediastinum. Most of the small cysts contained clear or only slightly haemorrhagic fluid and sometimes they appeared to project like small vesicles from a single or a group of lymph nodes or from the immediate surroundings of the lymph nodes.

Occasionally a palpable growth consisted partly of a blood filled cyst and partly of a solid tumour. The cyst and the tumour might have originated separately and later fused. Often however the tumour had obviously developed from the wall of the cyst (Fig. 7).

The incidence of sarcomas and cysts in the 4 experimental series is given in Table 1.

TABLE 1

No. ml. of rats	Age at time of inoculation (days)	Rats with sarcoma only	Rats with sarcomas and cysts	Rats with cysts only
7	3	3/7	0/7	0/7
10	4	6/10	7/10	0/10
3	1	2/3	0/3	1/3
13	1	4/13	6/13	7/13
		15/33	9/37	4/33

It is evident from the table that sarcomas and/or cysts developed in 28 of the 33 rats inoculated. Sarcoma developed in 24 (70 per cent) while 4 of the animals showed only cysts. Two of these 4 animals were however killed at 23 and 33 days respectively for histological study and it is possible that a sarcoma would have developed if the animals had been allowed to live longer. The rats with sarcoma which were not killed for histological analysis for serial passage or for human tumour reasons died within 6 weeks to 3 months of the inoculation presenting progressively growing tumours nearly always with metastases to the lungs. In one rat which died from pneumonia 62 days after inoculation the tumour in the thigh however was not larger than a pea.

Microscopical findings. As a rule the tumours resembled spindle cell sarcomas built up of densely crowded elongated cells arranged in an irregular network of bundles (Fig. 12). Two cell types could be dis-



Fig. 12 Histiocytic sarcoma built up of densely crowded elongated cells arranged in an irregular network of bundles. Htx-eosin $\times 100$

Fig. 13 Two types of cells can be distinguished in the spindle cell sarcoma. Htx-eosin $\times 500$

linguished (Fig 13) In one type the narrow nuclei were fairly rich in chromatin, cells were oblong and cytoplasm scanty The other type had oval or round vesicular nuclei, which optically were almost empty, the chromatin being concentrated along the nuclear membrane The nuclei had one or two distinct nucleoli The cytoplasm was most often relatively rich, pale, and slightly granulated The cells were round or fairly irregularly outlined and to a certain extent of a macrophage-like appearance Both types of cells were irregularly intermingled Numerous intermediate types were seen, and probably the cells represented different lines of development of one and the same type of cell Between the cells were sparse connective tissue fibrils, which, however, were only slightly, or not at all, fuchsinophilic On silver impregnation according to *Laidlaw* (Fig 14), they appeared distinctly As a rule, mitoses were fairly numerous

The slowly growing, hard tumours contained abundant interstitial fibrils and gave the impression of a rather highly differentiated fibro sarcoma

Softer, more medullary tumours contained an abundant admixture of the above mentioned cells with vesicular nuclei (Fig 15) Occasionally this cell type would predominate Sometimes the tissue was made up of rather large, detached, polygonal elements with eccentric nuclei and abundant cytoplasm showing central hyalinization and a peripheral basophilic, slightly granulated margin (Fig 16) Mitoses were common and sometimes of atypical appearance

The tumour tissue often showed numerous round or irregular necroses, containing detritus and more or less numerous nuclear fragments (Fig 17) In the margins of the necroses the tumour cells sometimes tended to be arranged in palisade fashion

The periphery of the sarcomas showed signs of invasive growth Strands of tumour cells were often seen growing between muscle bundles or individual muscle fibres Remnants of destroyed muscle fibres were observed in the outer layer of the tumour If the spine were invaded, the bone trabeculae showed lacunar destruction and degeneration of the cartilage

The metastases had, as a rule, the same histological appearance as the primary tumour

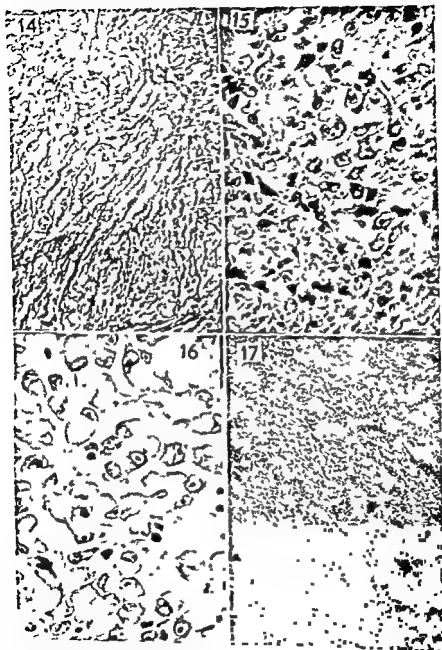
The lymph nodes showed all degrees of involvement, from marginal infiltration to complete destruction (Fig 18) Sarcoma cells were often

Fig 14 Silver impregnation reveals a fine network of fibrils in the sarcoma *Laidlaw* staining, $\times 140$

Fig 15 The picture of the medullary tumours is dominated by detached cells with vesicular nuclei Htx eosin $\times 500$

Fig 16 Part of the sarcoma is sometimes made up of fairly large cells with eccentric nuclei and abundant cytoplasm showing central hyalinization Htx eosin $\times 500$

Fig 17 Irregular band like necroses are often seen in the central part of the sarcomas Htx eosin $\times 40$



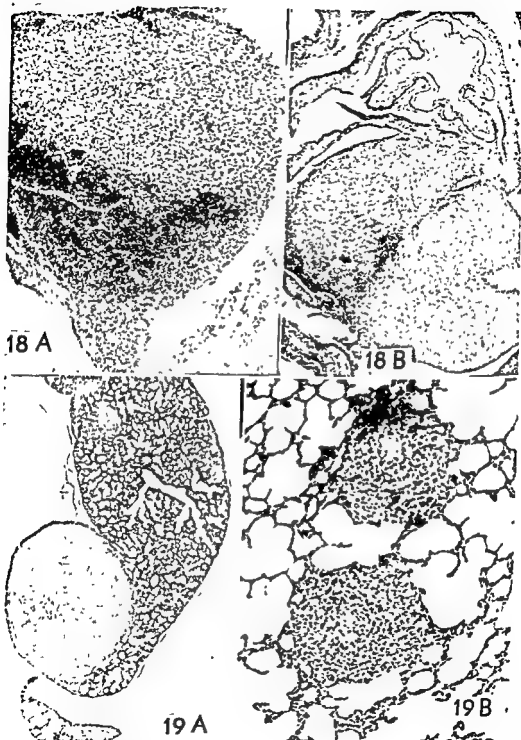


Fig 18 a Retroperitoneal lymph node with metastatic sarcoma Htx-cosin $\times 50$
 b Mediastinal lymph node completely obliterated by sarcoma. $\times 37$
Fig 19 a and b Metastases of the rat sarcoma in the lung Htx-cosin a $\times 14$
 b: $\times 100$



Fig 20 Wall of a recently developed cyst with a lining of swollen endothelial cells Htx eosin $\times 410$

Fig 21 A cyst wall with thin connective tissue excrescences enclosing vessels and lined by endothelial cells Htx eosin $\times 140$

Fig 22 Excrescences on the cyst wall partly lined by fibrin Htx eosin $\times 40$

seen including the surrounding fat tissue. Usually the spindle cell type would predominate but areas with detached rounded cells were also seen.

The metastases in the lungs were often of the spindle cell type, though as a rule more anaplastic than the primary tumour. They varied from single groups of neoplastic cells inside small vessels to

large, rounded or irregular foci (Fig 19). In some instances the metastases were situated peribronchially and some of these may have represented lymphogenic spread from lymph nodes in the mediastinum.

The lung tissue around the metastases was strikingly often haemorrhagic with a more or less abundant admixture of inflammatory cells. No cysts were found inside, or on the surface of, the lungs or in the mediastinum.



- Fig 23 Lymph node with distended sinuses and hyperplastic sinusoidal cells. A small cyst is seen close to the lymph node. Htx-cosin $\times 100$.
- Fig 24 The central part of the lymph node shows a cystic transformation. Htx-cosin $\times 100$.
- Fig 25 A small lymph node in the wall of a recently developed cyst. Htx-cosin $\times 145$.

The cysts usually had an extremely thin wall made up of connective tissue with an inner lining of a single layer of endothelium-like cells. They were sometimes flattened sometimes, particularly in recently formed cysts they were swollen with rounded, protruding nuclei, which not infrequently showed mitoses (Fig 20). Sometimes the endothelial lining of the cysts was multilayered. The cyst wall showed often thin connective tissue excrescences enclosing one or more vessels, and lined by swollen endothelial cells (Figs 21 and 22). Excrescences which had been cut transversely appeared to lie free in the lumen of the cyst. It is probable that the excrescences were remnants of the surrounding tissue which had been torn apart by the rapid expansion of the cysts. Occasionally the wall of the cyst was lined by a layer of fibrin. The inner surface of old cysts was smooth. The endothelium was lower, the cyst wall was thicker than in more recently developed cysts and, like the surrounding tissue, showed fairly rich deposits of blood pigment.

The cysts were of lymphogenic nature. Examination of young cysts revealed that they had developed in, or in the immediate neighbourhood of lymph nodes. The earliest demonstrable change appeared to be a widening of part of the intermediate or terminal sinus, where the endothelial cells were swollen and differed from the endothelium elsewhere in the sinus (Fig 23 and 24). At the same time the lymph vessels near the lymph nodes had widened and likewise showed endothelial hyperplasia. In the early stages of development, the cyst wall often contained a small lymph node (Fig 25), and the cyst lumen was occasionally in open communication with the dilated sinusoides in the lymph nodes.

The occurrence of cysts in connection with sarcoma was mentioned above. In one case this combination was seen histologically at an early stage. In that case the cyst wall showed a nodular thickening built up of fairly crowded parallel cells with oval or vesicular nuclei and scanty intercellular fibrils (Fig 26). The cells showed numerous mitoses and resembled those usually seen in the sarcoma. The picture strongly suggested an early sarcomatous transformation of the cyst wall.

In another case a lymph node adjacent to a cyst showed a marked proliferation of endothelial cells with vesicular, rounded or irregular nuclei. The lymph tissue was almost completely destroyed. The cells resembled the "microphage"-like cells seen in sarcoma (Fig 27).

2. Influence of the Age of the Rats

In the experiments described above rats were inoculated within at most 4 days of birth with the Schmidt Ruppin material. In 3 experimental series the susceptibility of older rats was studied. The results are given in tabular form below.

In the first series the animals were 12 days old. As in the previous series they were inoculated with 0.20 ml of a 1:5 sarcoma suspension subcutaneously in the back. The first tumours appeared after about one



Fig 26 Sarcomatous transformation of a cyst wall Htx eosin $\times 410$

Fig 27 a and b Marked proliferation of the sinusoidal cells in a lymph node adjacent to a cyst. The proliferating cells show a strong resemblance to the macrophage" like cells in the sarcomas Htx eosin a $\times 140$ b $\times 490$

TABLE 2

Number of rats	Age at time of inoculation (days)	Number of rats with sarcomas	Number of rats with cysts
11	12	10/11	0/11
11	23	3/11	0/11
5	full grown	0/5	0/5

month in 3 animals they were localized to the back in the others to the right thigh. The tumours often grew rapidly and 4 of the animals succumbed about 3 weeks later. Two of the rats showed metastases to the lungs in one rat the tumour had destroyed the spine with consequent paralysis of both hind legs and in the other the tumour had grown into the abdomen and metastasized to the omentum. The remaining rats were killed 7 weeks after inoculation. In these the tumours were rather smaller than the ones seen in newborn rats at corresponding intervals after inoculation no metastases were observed but they might have developed if the animals had been allowed to live longer.

The second series which consisted of eleven 23 day old rats received the same amount of sarcoma suspension into the right thigh. Tumours developed in only 3 of these about one month after inoculation but growth was relatively slow. In one of the animals gangrene developed in the right hind foot. Autopsy 3 months after inoculation revealed multiple metastases to the lungs and mediastinum. Of the other 2 animals one died 4½ months after inoculation with a large tumour on the back and metastases to the lungs the other, died 2½ months after inoculation a finger-size tumour in the right thigh and multiple metastases in the lungs were present.

None of the 5 adult full grown rats developed sarcoma. They were inoculated with 0.5 ml of a 1:5 suspension of minced tumour material and were observed for 5 months.

Hence it is evident from the foregoing that the Rous sarcoma material used can induce sarcoma not only in newborn rats but so though with decreasing frequency in rats up to 3-4 weeks of age. The interval between inoculation and appearance of tumours was the same as in animals inoculated in the neonatal period but growth and metastasization of the tumours seemed to be slower. The histological picture coincided in all essentials with the one described above but showed a predominance of spindle cells. It is noteworthy that no cysts occurred in any of the animals inoculated. Evidently this type of reaction occurs only in animals inoculated in the neonatal period.

1. Are the Rat Sarcomas Built Up of Rat Cells or of Transplanted Chicken Sarcoma Cells?

This question was studied serologically as well as by chromosome analysis of the rat sarcoma cells.

The serological analysis was kindly done by Assoc. Professor Anna-Brilla Laurell, Bacteriological Institute, Lund, using a micromodification of the double-diffusion-in-gel-method described by Wadsworth (1937). Antisera prepared by immunization of rabbits with cellular suspension of the chicken sarcoma was used. Extracts of a 2-month-old rat sarcoma were compared with identically prepared extracts from the chicken sarcoma and from normal chicken liver. Various dilutions of the extracts were studied.

A number of precipitation lines were observed in the spectra of chicken sarcoma—anti-chicken sarcoma serum and those of chicken liver extract—anti-chicken sarcoma serum. Most of the lines in these spectra showed reactions of identity.

No precipitation lines were found in the gel between the rat sarcoma extract and the anti-chicken sarcoma sera.

The results showed that the cells from which the rat sarcomas were built up were not of a chicken cell nature.

Chromosome analysis of the rat sarcoma cells performed by Professor A. Leván, Institute of Genetics, Lund, gave the same result. The chromosomes had the general appearance of rat chromosomes, but sometimes they differed in number from that in normal rat cells. The findings will be described in detail in a future paper.

4. *Induction of Rat Sarcoma and Cysts by Cell-Free Material from Chicken Sarcoma*

Four litters of newborn rats were inoculated with presumably cell-free supernatant from the Schmidt-Ruppin sarcoma material, homogenized, suspended in saline and centrifuged in the way described above. The results are given in tabular form below.

TABLE 3

Number of rats	Age at time of inoculation (days)	Rats with sarcomas only	Rats with sarcomas and cysts	Rats with cysts only
4	2	1/4	3/4	0/4
7	1	3/7	3/7	0/7
6	2	1/6	2/6	1/6
10	6	2/10	0/10	0/10

It is evident from the table that sarcomas as well as cysts developed in several animals. The number of positive animals was somewhat smaller than the number found after inoculation with cellular material. Sarcoma developed in 15 of the 27 animals. It was noteworthy that sarcoma was found in only 2 of the animals in the last series, in which rats were 6 days old at the time of inoculation. The most striking difference, however, was an increase in the interval between inoculation and the appearance of the sarcomas and cysts, *i.e.*, 2–4 months, as

against one month in the previous experiments. Once the sarcomas had appeared they grew more slowly and were of more fibrous consistency than in the previous experiments. The decreased aggressiveness was also reflected by the lower incidence of metastases. While almost all of the rats in which tumours were found in the earlier experiments developed metastases within one or 2 months, only few of the animals inoculated with cell free material had metastases within this time. Histologically the tumours usually had the character of cellular rather highly differentiated fibrosarcomas.

All attempts to induce sarcomas or cysts in newborn rats with Seitz filtrate from the Schmidt Ruppin sarcoma failed.

5 Serial Transfer of Rat Sarcomas in Rats

The chicken induced rat sarcoma could be easily transplanted to newborn rats. Growth was visible in all of the animals 4-7 days after the subcutaneous injection of 0.1 ml of finely minced 1:5 suspension of the rat tumour in Hank's solution. The transplants grew more rapidly than the original tumour and the hosts usually succumbed within 3 or 4 weeks. The tumours were usually softer than the original ones. They assumed considerable proportions and invaded the pelvis as well as the abdominal and thoracic cavities. They had a greyish white colour and the cut surface showed a tendency to undergo slimy or cystic degeneration. Metastases were often found in the lungs or mediastinum and sometimes also over the pleural surface or in the pericardium.

Microscopically the tumour was built up of densely packed cells with

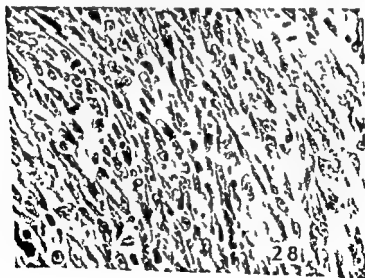


Fig. 28. Serially transplanted rat sarcoma. H&E stain. X 400.



Fig 30 a and b Chicken sarcoma induced by material from the ninth rat passage tumour. The sarcoma shows the usual picture of a Rous sarcoma and is built up of elongated cells and of detached "macrophage"-like cells a $\times 140$ b $\times 500$

the rat tumour was necrotic and the material transferred might have been taken from an unsuitable area of the tumour

The interval between the injection of Schmidt-Ruppin material from the chicken to the first rat and the transfer of material from the last rat in the series to the last two chickens was about 5 months. The inoculum from the first chicken consisted of 0.20 ml of a 1:5 suspension of sarcoma tissue. Each rat in the following series received 0.10 ml of

1 a rat sarcoma suspension and the amount of rat sarcoma inoculated to the chickens was in all experiments the same i.e. 1 ml of a 1:5 cellular suspension in Hank's solution. The duration of latency of the chicken tumour in the first 3 experiments was about the same as in the experiment where material from the ninth rat passage was used.

2 ml of the contents of a haemorrhagic cyst in a rat which had been inoculated 1½ months previously with Schmidt Ruppin sarcoma were injected into 2 chickens. Both were killed 3 months later and neither showed any pathological changes.

7 Occurrence of Hemagglutinins in the Rous Ruppin Sarcoma

To check the possibility of contamination of the sarcoma with polyoma virus the Schmidt Ruppin sarcoma was studied for haemagglutinins. mouse polyoma virus gives haemagglutination (HA) of Guinea pig and human O blood. The investigation was kindly done by Docent *Sven Bergman* Dept of Virology Lund.

Homogenized Schmidt Ruppin sarcoma was suspended 1:5 in saline and centrifuged for one hour at 13 000 rpm in an International Cold Centrifuge. The supernatant was carefully sucked off. Supernatant from sarcoma cells cultured *in vitro* was also used. The material was always used both in the fresh state and after heating to 56° C for 30 minutes before the test. HA tests were carried out on blood cells from guinea pigs, chickens, sheep and man. The specimens were incubated at +4°, +22° and +37° C for 24 hours and the results were read at 1, 2, 12 and 24 hours.

No haemagglutinins were demonstrable.

DISCUSSION

In the experiments here discussed the sarcomas developed quickest and most frequently in rats inoculated with cellular material from the chicken sarcoma. The possibility that the rat sarcomas may have been heterotransplants from the chicken sarcoma must therefore be considered. It is true that it would be a unique phenomenon in transplantation biology if cells from one class of animals should grow progressively in animals belonging to another class and the possibility is

therefore to find an sarcoma could be demonstrated in the gel precipitation test according to *Walsworth* and the chromosomes in the rat sarcoma cells had the appearance of rat chromosomes. The possibility of rats. The oma as excluded. The rat sarcomas must have

been induced *de novo* by some agent present in the chicken sarcoma used

In view of our earlier unsuccessful attempts to transfer Rous sarcoma to rats and mice, one might consider the possibility that the positive results were due to contamination of the Schmidt-Ruppin material with polyoma virus, for example, a possibility also suggested by Ising-Iversen. Polyoma virus-induced rat tumours are, however, of quite a different type, and have a latency of several months. In addition, no haemagglutinins could be detected in the extract of chicken sarcoma or in the supernatant from *in vitro* cultures of sarcoma cells, which would have been the case if the chicken tumour had contained polyoma virus.

Though the possibility of contamination with some unknown virus cannot be excluded with certainty, it is probable that the active agent was the same as the one inducing Rous sarcoma in chickens. It is true that no sarcomas developed in the rats inoculated with Seitz-filtered chicken sarcoma, but it is well known that filtration through a Seitz filter severely reduces the activity of Rous sarcoma material. Even in chickens it is sometimes difficult or impossible to transfer Rous sarcoma by filtrates. The best known case is the one reported by Gye & Andrewes (1926), who carried the Rous sarcoma through 9 successful cell passages in which filtrates were consistently negative. However, further investigations are necessary to ascertain whether the negative result with filtrates is due to too strong a reduction of the amount of virus. Svoboda *et al*, who also reported negative results of their experiments with virus extracts, discussed the possibility that a limited survival of transferred sarcoma cells permits for a gradual adaptation of the virus to the new hosts or that the transferred tumour cells induce a certain degree of tolerance to the Rous virus. This does not seem likely, as we did not succeed in eliciting sarcomas in rats with the Mill Hill material given in the same way as the Schmidt-Ruppin material.

It is not yet possible to decide with certainty whether we have to do with a variant (mutant) of Rous virus or whether the Schmidt-Ruppin strain is only a particularly virulent type of Rous virus. Much argues for the former possibility. In the previous article an account was given of comprehensive attempts to induce sarcoma in rats and mice with the Mill Hill strain of Rous virus. All of the attempts failed. The sarcomas induced in the chickens by the Mill Hill strain grew just as quickly as those induced by the Schmidt-Ruppin strain and both killed the birds after, roughly, equal intervals. In both investigations the tumour material was harvested at about the same time, *i.e.*, 8-12 days after inoculation. Both viruses induced tumours in all inoculated chickens. An additional finding against the assumption that the Schmidt-Ruppin strain might be only a particularly virulent form of Rous virus is the negative result of many previous attempts in various laboratories to transfer the Rous sarcoma to mammals (see preceding article). Neutralization tests with anti-Mill Hill serum as well as with anti-Schmidt-

Ruppin serum might be able to clarify the relation between the two strains used in our experiments. A thorough analysis of the strains of Rous virus used in various laboratories as well as their history seems also highly desirable.

Much suggests that the Schmidt Ruppin strain is identical with, or closely related to, the Rous strain used by Zilber *et al*, Svet-Moldavsky and by Svoboda *et al*. In the experiments carried out by Zilber *et al*, the rats presented only haemorrhagic cysts and multiple haemorrhages, whereas the other investigators reported a development of both cysts and sarcomas. The difference between our results and those obtained by these authors was mainly a matter of degrees of effectivity of the virus, as reflected in the frequency with which it induced sarcoma, and the period of latency. In our experiments sarcomas developed in 70 per cent of the inoculated rats within about one month, while Svet-Moldavsky reported the development of sarcoma in 3 out of 4 intra-embryonally inoculated rats within 6-7 months, and Svoboda *et al* in 2 out of 87 rats $4\frac{1}{2}$ and 7 months, respectively, after inoculation. It is not quite clear why the Schmidt-Ruppin material in Ising-Iversen's series of 12 newborn rats induced sarcoma in only 2 of the animals, and cysts in none. In her series the interval between inoculation and the appearance of the tumour was 3 months.

The similarity between the haemorrhagic cysts in the rats and the haemorrhagic disease which Rous or Fujinami virus produces in chick embryos and chicks (Duran-Reynals 1940) has been pointed out by Zilber *et al*, by Svet-Moldavsky and by Svoboda *et al*. But the differences are also of interest. Whereas in chicks the disease manifests itself in widespread and numerous blood blebs and extravasation of blood into the viscera, particularly into the liver and the spleen, the haemorrhagic cysts in the rats were localized to the region of the lymph nodes, especially in the groins, axillae, shoulders and neck, sometimes also in the pelvis and the retroperitoneum. The viscera remained unaffected. In chicks the virus thus appears to attack the vascular endothelium in general, in rats on the other hand, affecting only certain groups of lymph nodes and adjacent lymphatics. Zilber *et al* described the development of cysts as well as generalized bleeding, particularly in the lungs, in their rats. Their animals were inoculated during embryonal life, while ours received the inoculum during the first days after birth. It is possible that the extent of the vascular lesions varies with the age of the rats at the time of inoculation. Judging from our experiments, the virus will produce no cysts in animals 12 days of age or older at the time of inoculation.

According to Duran-Reynals (1940), the vascular lesions are manifestations of direct attacks by the virus causing necrosis of the endothelium and consequent bleeding. On examination of very recent lesions, he found that the endothelial cells occasionally were somewhat swollen. This swelling was more marked in rats in which the endothelial cells

in the affected lymph sinuses or lymph vessels were definitely enlarged often showing signs of proliferation. Thereby they differed distinctly from the endothelial cells in the unaffected parts of the lymphatic system. The necrotic effect on the endothelial cell by the virus on the other hand was but slight and can be a matter of fact only be presumed as a cause of the bleedings. The development of cysts may have been due to a combination of a toxic serous exudation in the lymph nodes and lymph vessels and a blockage of the efferent channels caused by proliferation of their endothelium.

That the development of cysts and sarcomas was ascribable to one and the same agent seems highly probable. In one case we found an early sarcomatous nodule which had developed in the wall of a cyst and in another the whole cyst wall showed sarcomatous transformation. Moreover in some lymph nodes with contiguous cysts the sinuses were filled with detached endothelial cells replacing the lymphatic tissue and having the same appearance as cells in the sarcoma. We could not demonstrate the virus in the contents of the cyst but *Sofi Moldavsky* found it in the cyst wall.

Microscopically it is possible to distinguish 2 types of cells in the Rous chicken sarcoma: an elongated fibroblast like type and a rounded macrophage like type. It has been shown in tissue cultures that both types of cells are reversibly interchangeable (*Doljansky & Tenenbaum* 1941). It was of interest to note that the rat sarcoma also showed 2 types of cells of a character similar to the one seen in chicken sarcoma. They were usually intermingled and both seem to possess malignant properties. In some areas or in some tumours one of the cell types would predominate. Soft tumours were as a rule built up mainly of detached macrophage like cells while the solid slow growing tumours usually consisted of elongated cells with fairly abundant interstitial fibrils. Both types of cells were seen in the metastases as well as in the serially transplanted tumours.

It might be argued that the tumour nodules in the lungs and other organs were not metastases caused by disseminated sarcoma cells but induced *in loco* by virus brought there by the blood or lymph stream. It is difficult to exclude this possibility. Yet it is striking that the rats which were killed early never showed any tumour nodules in the lungs or lymph nodes while growths in these organs were more or less the rule in animals allowed to live longer. The nodules in lungs and lymph nodes were always much smaller than the tumours at the site of injection and showed the same pattern of distribution as the one usually seen on dissemination of tumour cells. Disseminated sarcoma cells were frequently seen in the small vessels of the lungs.

It is of a considerable interest to note that the virus induced rat tumours could be transferred further in rats only by material containing living tumour cells. All attempts to transfer the rat tumour to new rats by means of cell free material failed and without knowledge of the

history of the rat sarcomas nobody would suspect them of being virus induced. The virus could be demonstrated by re transfer of tumour material to chickens which could be done successfully not only with material from the first virus induced rat sarcoma but also with material from successive generations of transplanted tumours in rats. Since the capacity of the rat tumours to induce sarcoma did not appear to decrease on passage from rat to rat the virus apparently multiplies in the rat cells, a view already put forward by *Spoboda et al* (1961) who recovered the virus from the 21st rat passage. The form in which the virus is present in the rat sarcoma can at present only be conjectural. The virus in the rat cells may be masked or the amount of the virus in the rat sarcoma is not large enough to evoke sarcomas in new rats. Elucidation of this problem is urgently required.

SUMMARY

Newborn rats were inoculated subcutaneously with Rous virus (Schmidt Ruppian strain). After an interval of about one month progressively growing sarcomas developed at the site of injection in about 70 per cent of the animals and subcutaneous cysts in about 30 per cent. The cysts were as a rule filled with blood stained serous fluid and were usually present in the groins, axillae, shoulders and neck, sometimes in the retroperitoneal tissue or in the pelvis. The cysts were of lymphogenic nature and their development could be followed from dilated lymph sinuses or from lymph vessels in the neighbourhood of lymph nodes. The sarcomas often had the characteristics of spindle-cell sarcomas of varying degrees of maturity. Metastases occurred in the retroperitoneal and mediastinal lymph nodes, in the lungs and in the pleural cavities. Some of the sarcomas developed from cyst walls. Not only rats inoculated in the neonatal period but also animals inoculated at 3 weeks of age developed sarcoma at the site of injection of Rous sarcoma material. Haemorrhagic cysts appeared only after inoculation of newborns. Sarcomas could be induced not only by cellular but also by presumably cell-free material from the chicken sarcoma. Serial transfer of the rat sarcoma in young rats offered no difficulties. After 4 passages in newborn rats the tumours could also be successfully transplanted to 19 day old rats and finally also to one month-old rats. All attempts to transfer the rat sarcoma to other rats by means of cell free material from the rat tumour failed. On the other hand the tumours could be successfully transferred to chickens which 4-6 weeks later showed a Rous sarcoma at the site of inoculation. This was possible not only with material from the first chicken induced rat sarcoma but also with material from series transplanted rat tumours.

It is concluded that the strain of Rous virus used in the experiments differs from the classical strain in its behaviour on inoculation into rats and has the capacity to induce sarcomas not only in birds but also in mammals.

THE LIVER AND PANCREAS IN ACUTE ETHIONINE INTOXICATED RATS

Correlation of Morphology to Metabolism of the Liver

By

Y. FOLUND

Received 30.1.61

α -Ethionine (α -amino γ -ethylmercaptobutyric acid) an amino acid analogue of methionine has been used during the last decade mainly in experimental research in pancreatic function. A number of investigations have revealed that ethionine administrations gives rise to lesions of the pancreas (1, 7, 15, 18) so called "ethionine pancreatitis" (8, 9). The biochemical effect of ethionine may be due to (i) interference with the intermediate methionine metabolism (12, 16) (ii) interference with the metabolism of amine-containing compounds (4) and (iii) incorporation into abnormal cellular proteins (4). Interestingly by creating amino acid imbalance a high dietary methionine intake will produce pancreatic lesions resembling those induced by ethionine (10).

Ethionine administration in the first place affects organs with a high protein turnover such as the liver and pancreas. It has been found that ethionine upsets the metabolism of the liver in starved rats, the glycogen level being markedly depressed (11, 13, 14) and the total fat content increased (5, 6).

We have deemed it of interest to combine electron microscopical investigations of the rat pancreas in acute ethionine intoxication (3) with studies on the metabolism of the liver. Additional reasons why these liver studies were considered desirable are that previous morphological investigations have mostly been made on animals subjected to prolonged ethionine administration (5, 17, 18) and that the metabolic studies have been performed on starved animals.

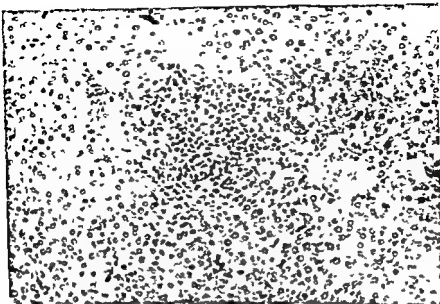
The present report describes the microstructure of the pancreas and liver in non-starving rats exposed to acute ethionine intoxication. The glycogen, total fat and protein contents of the liver have been estimated and correlated to the morphological observations.

MATERIALS AND METHODS

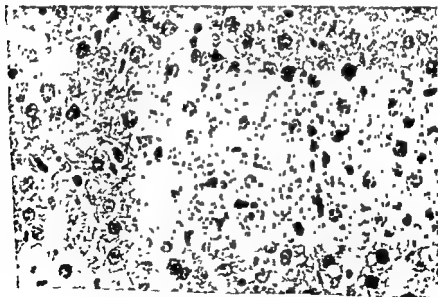
The experiments were carried out on 200 g. They were divided into two groups. The rats were divided

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*Fig. 1*

Focal necrosis centrally and hepatic cells containing acidophilic bodies H & E.

*Fig. 2*

Small vacuoles in the cytoplasm of liver cells H & E.

doses of ethionine by stomach tube and were decapitated after 3 days. The 4th group comprised 10 untreated controls.

Group I included 17 rats, 7 of which received a single ethionine dose of 200 mg per 100 g body weight, the other 10 being given the same dose on consecutive days. These rats were killed 48 and 24 hours respectively, after the last dose. *Group II* comprised 23 rats having daily dosages of 100 mg per 100 g body weight for 2 days. Of the 9 animals in *Group III* 4 received 75 mg and 5 received 50 mg of ethionine per 100 g body weight daily for 2 days. The rats in both of the latter groups were sacrificed 24 hours later.

The liver and pancreas from all of the rats were examined under the light microscope, the amounts of glycogen, total fat and nitrogen were estimated in 42 of the 59 livers.

Microscopical

Some pieces of liver were fixed in 10 per cent formalin solution and stained for fats (with scarlet red), some in Zenker's solution and stained with hematoxylin-eosin and some in 95 per cent ethanol and stained by Best's technique. Pieces of pancreas intended for staining with hematoxylin-eosin were fixed in formalin and those to be stained according to Lane-Hensley in 2.5 per cent potassium bichromate followed by a saturated alcoholic (95 per cent ethanol) solution of mercuric chloride.

Chemical

Immediately after the rat had been killed a piece of liver weighing some 200 to 500 mg was dissected and placed in a 5 per cent trichloroacetic acid solution for determination of extractable and non-extractable glycogen fractions by van der Vies's method (19) in slightly modified form (2). The remainder of the liver—after saving some fractions for microscopical examination—was immersed into 5 ml of distilled water weighed and homogenized. A portion of the homogenate (containing approximately 20 to 30 mg of fats) was extracted with an aliquot of a 3:1 alcohol-ether mixture. The extract was evaporated down to dryness under vacuum and the residue dissolved in chloroform. After filtration the chloroform fraction was evaporated down to dryness and the total fat content determined by weighing. The total nitrogen content of the homogenate was estimated according to Kjeldahl; the value thus obtained being multiplied by 6.25 to provide a calculated nominal protein content.

RESULTS

Microscopical

Group I (200 mg of ethionine per 100 g body weight). The rats receiving (one or two) ethionine doses of this magnitude were all seriously affected and showed a high mortality. The liver exhibited a distinct, pale, yellow discolouration, the pancreas had a 'doughy' appearance and was of a white hue.

In some lobules of the liver were central necrotic foci with invading round cells and polymorphonuclear leukocytes, in others similar foci were located farther peripherally (Fig. 1). Small vacuoli often occurred marginally in the cytoplasm of the liver cells, the entire cytoplasm of some cells was occupied by large vacuoli (Fig. 2). All livers were free from glycogen crusts, and no sudanophil material was observed at the site of the vacuolines in slides stained for fats. Fatty deposits were present in the majority of livers in the form of minute to large droplets diffusely scattered throughout the lobules. Some of the liver cells contained strongly acidophil, globular formations which here and there had been 'expelled' from the liver cell into the sinusoids. Furthermore Kupffer cell proliferation (Fig. 3) was observed and, in the portal

canals, distinct increases of lymphocytoid elements and plasma cells

The most characteristic change in the pancreas was vacuolization of the cytoplasm in the exocrine cells (Fig. 4). Small vacuoles were located basally. Large vacuoles with a honeycomb appearance often occurred, occasionally occupying the major portion of the cell cytoplasm. Small accumulations of an acidophil, granular substance were seen in the vacuoles. Such parts of the cells as bordered on the lumina contained an abundance of zymogen granules. The basal parts of the cells exhibited diminished basophilia. Neither interacinous nor interlobular changes were observed.

Group II (100 mg of ethionine per 100 g body weight). The changes encountered in the livers and pancreases from these rats were similar in principle to those seen after the larger dose (200 mg) although they were less marked and acidophil degeneration was absent in the liver. Some livers merely exhibited slight cell proliferation in the portal canals. No fatty deposits were present, and in such livers in which glycogen crusts occurred these were confined to cells located centrally in the lobules.

Group III (75 and 50 mg of ethionine per 100 g body weight). In this group the liver changes were negligible: slight cell proliferation in the portal canals and small cytoplasmic vacuoles in some cells. The majority of livers presented no morphological abnormalities. The changes in the pancreas were also very discrete, taking the form of delicate vacuolization of occasional acinous cells. The microstructure of most pancreases was normal.

Chemical

Mean glycogen, protein, and total fat contents in per cent of wet liver weight are recorded in Table 1. The table reveals that after exposure to the higher ethionine dosages (Groups I and II) 6 livers of 23 were completely devoid of glycogen and the others had extremely low glycogen levels. After lower doses (Group III) the glycogen levels showed a moderately significant decrease as compared with those in the controls.

TABLE I
Mean Glycogen, Protein, and Total Fat Contents (in Per Cent of Wet Weight) in Livers from Acutely Ethionine Intoxicated Rats

Group	Dosage mg/100 g	No. of Rats	Glycogen		Protein $\bar{x} \pm s_x$	Total Fat Content $\bar{x} \pm s_x$
			Extractable $\bar{x} \pm s_x$	Nonextractable $\bar{x} \pm s_x$		
I	200	8	0.04 \pm 0.01*	0.03 \pm 0.01	17.60 \pm 0.49	12.19 \pm 1.47
II	100	15	0.10 \pm 0.03§	0.07 \pm 0.02	18.82 \pm 0.31	7.47 \pm 0.21
III	50 and 75	9	3.07 \pm 0.43		17.13 \pm 0.31	7.12 \pm 0.26
IV		10	4.96 \pm 0.71	0.23 \pm 0.02	16.81 \pm 0.47	6.40 \pm 0.27

* 1 zero glycogen estimation.

§ 5 zero glycogen estimations.

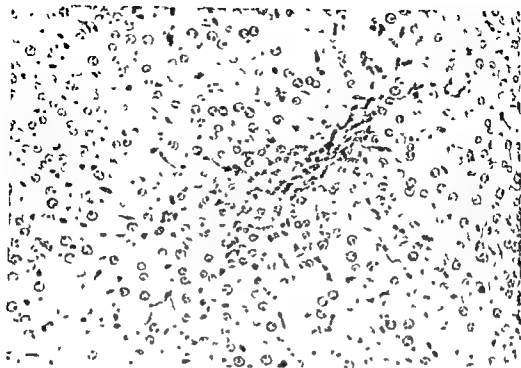


Fig 3
Proliferation of kupffer cells H & E

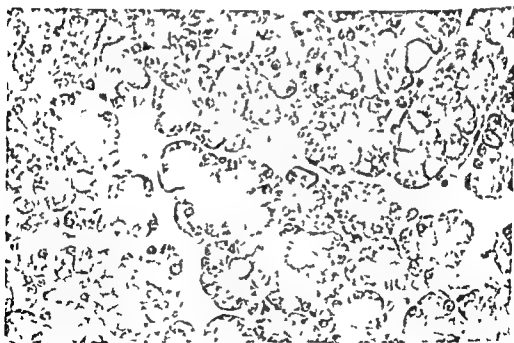


Fig 4
Vacuolization of exocrine pancreatic cells H & E

and correlated to the morphological observations 10 untreated rats were used as controls. The rats were killed 3 days after the first ethionine dose had been given by stomach tube. The rats were divided into 3 groups according to dosage viz 200 mg per 100 g body weight (Group I), 100 mg per 100 g (Group II), and 75 or 50 mg per 100 g (Group III). The investigation gave the following results:

1. Vacuolization of liver cells and of acinous pancreatic cells was observed in all of the ethionine treated groups, the changes being more marked after higher doses. The higher doses produced necrotic foci and acidophil degeneration in the liver.
2. Ethionine interfered with the carbohydrate metabolism, causing a reduction of the glycogen content of the liver. After high doses (200 and 100 mg per 100 g) several livers were entirely devoid of both extractable and non extractable glycogen. Whilst the total fat content of the liver had distinctly increased after the highest dose (200 mg per 100 g), the liver's protein content remained unaffected in all dosage groups.
3. The degree of morphological changes in the liver was parallel to the disturbances of the carbohydrate and fat metabolism.

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Small amounts of non-extractable glycogen occurred in the livers with extractable glycogen

The protein content of the liver showed no alterations in the three groups, the values in all of them being within the range of normal variation. On the other hand, whilst the content of total fat was markedly enhanced in Group I, the amount of fat was normal in Groups II and III.

COMMENTS

The present investigation confirms earlier findings (1) in showing that lesions of the liver and pancreas incurred by intoxication with the "foreign" amino acid ethionine are dependent on the magnitude of the dose, and it also verifies a previous report (11) in not disclosing any sex differences. Large doses have in common that they in both organs give rise to a formation of vacuoles in the cytoplasm, predominantly confined to such parts of the cells as border on the blood capillaries.

In some of the animals receiving the highest ethionine dose (200 mg per g body weight) the liver exhibited more serious lesions than the ones seen in the pancreas, namely cellular necrosis and so-called acidophil degeneration. Such liver lesions are typical occurrences after exposure to toxic agents of various kinds.

The chemical findings provide further evidence of the fact that large ethionine doses have a highly toxic effect on the liver. Thus, just as previous investigations have shown in starved rats, the present study on unstarved rats receiving large doses (200 mg per 100 g) revealed that the liver's fat content was increased and its glycogen content distinctly reduced. When the ethionine dose was reduced to 100 mg per 100 g the liver glycogen levels remained low but the fat content of the liver remained unaffected. Following a further diminution of the dose to 75 or 50 mg per 100 g there was an insignificant lowering of the liver glycogen level. This indicates that there is a fairly well-defined transition towards a more marked ethionine action on the carbohydrate metabolism—somewhere between 75 and 100 mg per 100 g body weight under the experimental conditions obtaining in the present investigation. The response of the liver to a large ethionine dose in the form of reduced glycogen content and increased fat content is the same as when other toxins—such as carbon tetrachloride or chloroform—are administered. The complete absence of glycogen, even of so-called bound or non-extractable glycogen, in some of the rat livers is remarkable. Complete disappearance of liver glycogen after administration of other types of toxic agents has apparently not been reported.

SUMMARY

The morphology of the liver and pancreas from 49 rats exposed to acute ethionine intoxication was studied under the light microscope. The glycogen, total fat, and protein contents of the liver were estimated.

MICROCIRCULATORY ALTERATIONS IN BILIARY OBSTRUCTION

By

YACUF EDLUND and LARS ERIK GELIN

Received 30 vi 61

Biliary obstruction is a common and severe clinical condition, known to result in morphological and functional disturbances of the liver. Both peripheral and central necrosis occur in the liver lobules. Proliferation of the bile-ducts and "cell-unrest" develop. These changes occur both in man and animals, most rapidly in rats. The genesis of the necrosis is, however, still obscure.

The purpose of this study was to correlate microcirculatory and histological changes in the liver in experimental biliary obstruction, in order to evaluate the genesis of such necroses.

MATERIAL AND METHODS

25 white rats weighing about 200 g were subjected to ligation of the common duct close to the liver. 10 rats died late after this procedure and were excluded from further studies. 3 rabbits weighing 2.5 kg were subjected to the same procedure.

The animals were then taken at different times after the ligation of the common duct for studies according to the following:

- | | |
|----------|---|
| Group I | 2 and 6 days after ligation (3 respectively 4 rats) |
| Group II | 8 days after ligation 2 rats and 1 rabbit |
| | 10 days after ligation 3 rats |
| | 15 days after ligation 3 rats and 2 rabbits |

The animals were anaesthetized with ether Nymal. A small subcostal incision was made to expose the liver under a Leitz Ultrapak vitalmicroscope equipped with electronic flush for photographic recording. After the vitalmicroscopic investigation a biopsy was taken from the liver for histological investigation and the animals sacrificed.

RESULTS

Biliary Obstruction of 2 and 6 Days Duration

Seven animals were examined two and six days after ligation of the common duct.

Gross observations. The liver was slightly enlarged, firm, pale, with rounded edges. The surface was even. The common duct was dilated proximal to the ligature.

Vital microscopic findings. In the periphery of the liver lobules pale and yellow areas were regularly found. Within these areas haemor-

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MICROCIRCULATORY ALTERATIONS IN BILIARY OBSTRUCTION

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JANNE EDLUND and LARS-ERIK GELIN

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The purpose of this study was to correlate microcirculatory and histological changes in the liver in experimental biliary obstruction, in order to evaluate the genesis of such necroses.

MATERIAL AND METHODS

25 white rats weighing 300-350 g were used. The animals were divided into two groups according to the following:

- | | |
|----------|---|
| Group I | 2 and 6 days after ligation (3 respectively 4 rats) |
| Group II | 8 days after ligation 2 rats and 1 rabbit |
| | 10 days after ligation 3 rats |
| | 15 days after ligation 3 rats and 2 rabbits |

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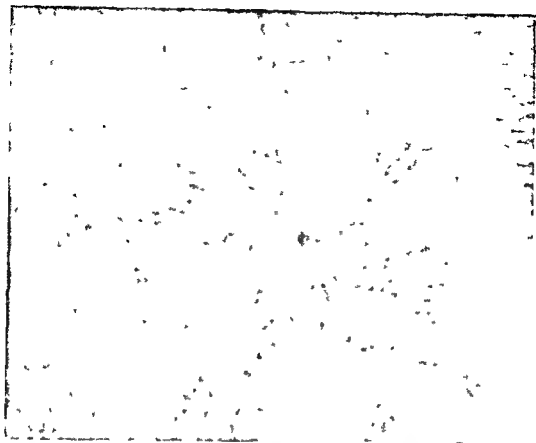


Fig 1

Vitalmicroscopic picture from the liver 2 days after biliary obstruction. Note the pale necrosis in the periphery of the lobule.

rhages were occasionally found. The microcirculation showed the following characteristics: within the portal venules the flow of blood appeared to be even and rapid; in the sinusoids the red cells were aggregated and especially these sinusoids near the pale and yellow areas showed stagnation of aggregated red cells and white masses. In the central veins a retardation of the flow of cells was observed. The central veins contained aggregated red cells and white masses. There were, however, no occlusions of the central veins (See Fig 1 and 2).

Histological findings. The histological picture was about the same in all animals. Two different necrotic areas within the liver lobules could be recognized: one in the periphery of the lobules containing lymphocytes and plasma-cells and also leucocytes (see Fig 3); another necrotic area was recognized in the central part of the lobules containing lymphocytes, leucocytes, plasma cells, and red cells. Proliferations of the bile ducts and round cell infiltration in the portal canals were also found. Within the necrotic areas an accumulation of a granular and strongly acidophil substance was found.

The observations were interpreted in the following way: Early after biliary obstruction pale necrosis is formed in the periphery of the

1
4

Fig 2

Valm. c p c p ture from the 1 or 6 days after blar. obstruct on. Note the pale necro. s. n the p r p h e y and the stagnat. n of aggregated cells n s n u s o d s an i s t a s in the central cin.

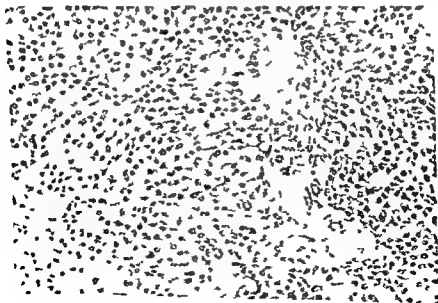


Fig 3

Sec. t c area in the periph. v. f the 1 or 11 l u s 2 l a s of blar. obstruct. n

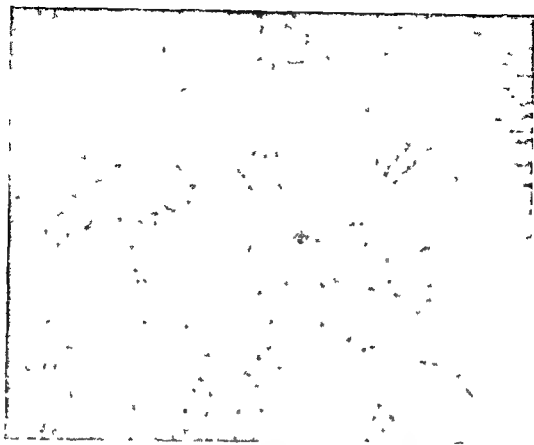


Fig 1

Vitalmicroscope picture from the liver 2 days after biliary obstruction. Note the pale necrosis in the periphery of the lobule.

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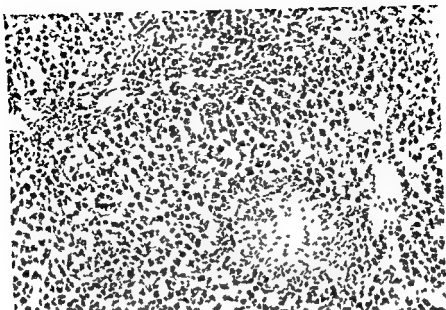


Fig. 5

Necrotic area in the central part of the liver lobule 8 days of biliary obstruction

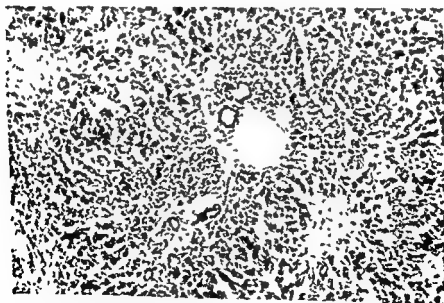


Fig. 6

Disturbed architecture of the liver lobules with proliferation of fibroblasts intra-lobularly and in the portal canals 10 days of biliary obstruction

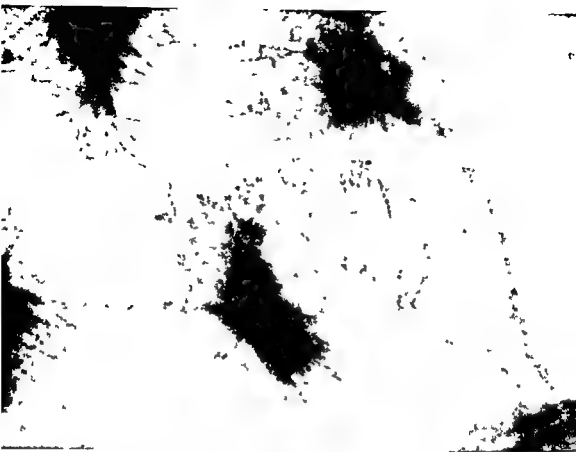


Fig. 4

Anastomosis between portal venule and central vein—Fek's fistula—from an animal 15 days after biliary obstruction. Note also the stagnation of cells in dilated sinusoids.

lobules. Aggregation and tendency to stagnation of blood cells occur in the central parts of the lobules, later resulting in haemorrhagic necrosis.

Biliary Obstruction of 8-15 Days Duration

8 rats were examined 8, 10 and 15 days after ligation of the common duct. 3 rabbits were examined in the same way after 8 and 15 days.

Gross observations. The liver was enlarged, firm, pale and showed a granular surface. The common duct was markedly dilated.

Vital microscopic observations. The borders of the liver lobules were less sharp than in group I. Pale and yellow areas in the periphery were regular findings. These areas contained small haemorrhages. In the central parts of the lobules haemorrhages were common. The micro-circulatory pattern was more markedly changed here than in group I. Aggregation of blood cells was present both in portal venules, sinusoids and central veins. The sinusoids were dilated and contained occlusive aggregates of blood cells. The flow rate of blood in the central venules was very slow and the venules were sometimes occluded. Occasionally microthromboses were found in the central and sublobular veins. Be-



Fig. 7

Areas of necrotic liver cells in the periphery of liver lobules 4 hours after intrachylemic infusion of 0.3 ml of sodium taurocholate (2 per cent)

seems unreasonable to assume these necroses to be of hemodynamic origin.

The necrotic areas around the central veins were increasing in number with time after ligation. Necroses around the central veins have circulatory genesis (Himsuorth 1954). It thus seems likely that these two types of necroses are of different origin.

The complex morphology and circulatory pattern of blood within the liver has been extensively studied by Knisely, Bloch & Warner 1948, clarifying the relationship between arterial, portal, sinusoidal and hepatic blood flow in physiology which is the basis for the understanding of the microcirculation here discussed.

The central necroses are haemorrhagic and occur later than the peripheral ones. They develop at a time when a disturbed flow of blood is apparent within the sinusoids and efferent venules. Central haemorrhagic necrotic areas have also been found in induced aggregation of blood cells (Lajers & Gelin 1951) which is in support of the contention that the central necroses found in these experiments are related to the disturbed flow with anemia from stagnated aggregated red cells.

In portal and biliary obstruction regenerative and degenerative processes alternate. The formation of connective tissue in the portal canals

tween the portal venules and the central veins some sinusoids were dilated two to three times and transformed to shunts. These shunts were 50–100 μ in diameter in animals in which biliary obstruction had lasted for 15 days (see Fig. 4).

Histological findings As in group I peripheral and central necrotic areas were found in the liver lobules (Fig. 5). The portal canals were dilated from proliferation of bile ducts and round cell infiltrations, mostly leucocytes. A proliferation of fibroblasts and formation of collagen was recognized in the portal areas. This proliferation migrated into the lobules causing a break in the liver cords with isolation of liver cells which in some places became necrotic (Fig. 6). A "cell unrest" was apparent i.e. enlarged liver cells, nuclear polymorphism with nucleolar enlargement, increased number of mitoses and appearance of binuclear liver cells.

The central haemorrhagic necrotic areas contained leucocytes and fibroblasts. The observed changes were most pronounced after long-standing obstruction.

Rabbits were observed in the same way as the rats and showed the same microcirculatory and histological changes. It was also possible to observe the capillary flow in the untouched bulbar conjunctiva. This showed marked aggregation of blood cells with a tendency to obstruct the venules by settling.

The experiments on rabbits showed that not only a local but also a general aggregation of blood cells was present.

DISCUSSION

A disturbed capillary flow within the liver after common duct ligation on rabbits was described 1927 by *Loeffler* using vital microscopy. He observed early, pale and red, necrotic areas in the lobules but did not differentiate between the genesis of the two types of necrotic areas. Our experiments make clear that following ligation of the common duct a pale and yellow necrotic area is formed in the periphery of the lobules and a haemorrhagic necrotic area is formed in the central part of the lobules. According to our observations, the peripheral pale necrosis occurs earlier than the central haemorrhagic necrosis. This is also in accordance with earlier studies few hours after ligation of the common duct (*Edlund* 1948).

The peripheral necroses are of a pale yellow colour indicating that bile leaks into this area. In accordance to *Ohno* 1931, *Cameron & Oakley* 1932, *Kikuchi* 1935, the peripheral necroses are caused by bile leaking from the interlobular bile ducts or the canals of Hering. Intracholecystic infusion of sodium taurocholate also produces such pale peripheral necroses. This favours the assumption that bile acid is the toxic agent (see Fig. 7). The flow in the portal venules and the peripheral sinusoids was undisturbed at the time for the genesis of these necroses, thus it

and intralobular signifies an early cirrhosis. In this stage abnormal anastomoses between the portal and hepatic venules are built up from transformed sinusoids (widened by shrinkage of the lobules) acting as Eck's fistula.

A marked and increasing aggregation of blood cells is also apparent in the general circulation. The causes of aggregation observed in these experiments must be discussed on a local and general basis. Within the first days of ligation aggregation only appeared in the efferent part of the sinusoids and in the central veins but not in the portal venules. It is probable that this early aggregation of cells in the efferent venules is produced by substances or metabolites from regurgitated bile or injured liver cells. The circumstance that the aggregation is first observed in the efferent venules is, however, also an incident occurring in capillary beds of less complicated morphology, i.e. the bulbar conjunctiva network (Knisely 1947, Gelin 1956). No conclusion can be drawn from this observation concerning the genesis of this aggregation.

The fact that most of the early observed central necroses belong to the same lobule which contain peripheral necroses, however, support the assumption that a local stagnation of aggregated blood cells is responsible for the central necroses, hence that this early appearance of aggregation of blood cells is induced by local and not by general factors.

The aggregation of blood cells appearing in the portal venules and in the general circulation later in the course of bile duct obstruction, can be fully explained by changes in the plasma protein pattern with accumulation of large and viscidizing protein molecules causing an increased ESR which regularly develops parallel with time after obstruction of the common duct.

In the sinusoids aggregation is present after 8 days. This aggregation was severe enough to plug some of the sinusoids. The flow was always slow in the sinusoids. A slow flow in itself can accelerate aggregation if the blood is prone to aggregate the cells, but not if the quality of the blood is normal.

From our observations we assume that the early aggregation is induced by substances from the early peripheral liver cell damage initiated by bile leak.

In our opinion the aggregation, present in portal venules and general circulation, is ascribable to changes in the plasma leading to a decreased suspension stability of the blood.

SUMMARY

Ligation of the common duct changes the microcirculatory and histological pattern of the liver in rats and rabbits. A marked and progressive aggregation and stagnation of cells occur starting in the sinusoids and central veins. Later a generalized aggregation and stagnation of blood cells was observed.

On the basis of the good correlation found between vital microscopic and histological changes we propose

that these flow-changes of blood are initiated from injured liver cells caused by bile leaks (bile acids) in the periphery of the lobules;

that the stagnation of aggregated cells results in central haemorrhagic necroses

Signs of early cirrhotic changes with the appearance of portal caval shunts (Fick's fistula) arise in prolonged biliary obstruction

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ULTRASTRUCTURAL CHANGES IN THE SENSORY NERVE FIBRES IN THE SKIN OF THE FROG (*RANA* *TEMPORARIA*) AFTER CIRCUMSCRIPT IRRADIATION WITH Po^{210} α -PARTICLES (5.3 MeV)

By

A. M. ISONÄKÄ¹, R. M. BERGSTROM and I. KIVIMO

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Most of the studies concerning the effects of ionizing radiation on the nervous tissue deal with effects of large doses of irradiation, which result in severe destruction of the morphological structures of the cell material (Janzen & Warren 1942) and depression of the physiological functions (Yamashita & Miyake 1952, Gerstner, Orth & Rickey 1955). Recently, however, Bachofer (1957) and Bachofer & Gautieriaux (1959) have reported that the initial effect of x-rays on the function of the peripheral nerves in the rat and on the giant nerve fibre in the earth worm results in an enhanced electrical activity, i.e. an increase in action potential and its propagation velocity. In order to study these effects of ionizing radiation on the electrical activity of nerve cells, a method was developed (Bergstrom, Blafeld & Brenner 1960) using a cylindrical Polonium²¹⁰-source (supplied by the Radiochemical Centre, Amersham, UK) for circumscribed irradiation of single nerve fibres with α particles (energy 5.3 MeV, nominal strength of the source at the time of the experiments 0.4–1.1 mc, half-life 138.4 days). The method used as well as the effects of the α particles on the electrical activity of single nerve fibres of the frog has been presented elsewhere (Bergstrom, Blafeld & Brenner 1960). This study is concerned only with the ultrastructural changes of the nerve fibres irradiated with Polonium²¹⁰ α particles.

METHODS

Sensory nerves obtained from the dorsal skin of healthy frogs (*Rana temporaria*) were used in the experiments. The nerves were excised from the decapitated animals and placed in oxygenated Tasaki Ringer solution (Tasaki 1939) for further preparation. The isolation of single nerve fibres was performed in the solution at

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18° C without distending the material. For this procedure the nerve was mounted in a specially made perspex case which could be lifted to the source of irradiation.

The experiments were made as short as possible in order to avoid structural

from 1000 to 4000 rep/min. Doses varying between 750 and 34 500 rep were used. The distance between the source of irradiation and the nerve fibre was adjusted so that the whole axoplasm was penetrated by α particles.

Unirradiated fibres treated under the same physiological conditions as described above served as control material.

Some cutaneous nerves of normal frogs were also removed after being fixed alive and examined as normal control material.

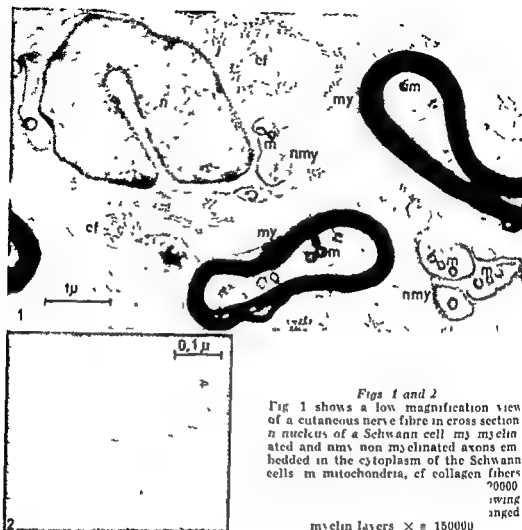
Technique for Electron Microscopy

The nerve fibres were removed immediately after the experimentation and immersed into 1 per cent osmium tetroxide solution prepared according to Björstrand (1953) and then rapidly cut into small sections and fixed for 2 hours in the same fixing solution which was changed three times. After fixing the pieces of tissue were rinsed for 15 minutes in Tyrodes solution, rehydrated in graded methyl alcohol series and embedded in methacrylate mixture or in Araldite. Sections were cut with a Porter Blum ultramicrotome and examined under an Akashi Transscope TRS 50 FI electron microscope. Several hundred exposures were made using primary magnifications up to 30 000 times.

RESULTS AND DISCUSSION

The normal skin nerve fibres of the frog consist of both myelinated and non myelinated axons embedded in the Schwann cell cytoplasm and of supporting peri- and endoneurial connective tissue (Fig 1 and 2). In the last years the finer structure of peripheral nerves has been to a large extent (see e.g. the comprehensive review of Fernandez Moran & Brown 1958).

Due to the experimentation used in this work the results obtained are almost purely degenerative in their nature thus making it difficult to analyse the findings and particularly to distinguish preparative artefacts from the changes caused by irradiation. Consequently the results obtained do not allow for such detailed conclusions as were desired as regards the relationship between ultrastructural changes and physiological alterations. In general the degree of changes in the different experimental groups clearly shows a positive correlation to the corresponding doses of irradiation. A quite remarkable variation of findings in different nerve fibres within a certain group can be found particularly in the group given the smallest dose of irradiation (750 rep). The findings may be summed up under following subheadings.



Figs 1 and 2

Fig 1 shows a low magnification view of a cutaneous nerve fibre in cross section. *n* nucleus of a Schwann cell, *my* myelinated and *nmy* non myelinated axons embedded in the cytoplasm of the Schwann cells, *m* mitochondria, *cf* collagen fibers

20000

swelling

induced

myelin layers $\times = 150000$

A⁻ Schwann Cell Cytoplasm

The most striking phenomenon even after the smallest dose of irradiation (750 rep) is the intensive swelling and vacuolisation of the cytoplasm of the Schwann cells. It is most clearly seen in those portions of the Schwann cells, which have only non myelinated axons embedded in their cytoplasm and in which some of the free cytoplasm is already visible in the normal material. After the bigger doses (over 10 000 rep) the cytoplasm almost completely loses its structure containing few granules (Fig 6) or granular masses, and where some remnants of swollen and vacuolated mitochondria can occasionally be observed.

Most of the vacuoles have a pronounced osmophilic boundary connecting them to the cell membrane. Profiles of this kind are interpreted as remnants of non myelinated axons embedded in the cytoplasm. The entire axoplasm is destroyed and the vacuoles are filled in most cases with a loose, granular mass, where fragments of the mitochondria or small clusters of axoplasmic remnants sometimes are seen.



Fig 3

swollen and fragmented mitochondria or groups of small vesicles which are fragmentation products of the ergastoplasmic channel system. At the arrow branching of the fibre \times ca 15000

Accompanying the swelling phenomena of the Schwann cell cytoplasm are some changes occurring in the irradiated material. These apparently affect the myelin structure as well as the axon. As shown by *Robertson (1958)* and *Engstrom & Wersall (1958)* components of the Schwann cell cytoplasm can be observed between the myelin arches



Figs 1 and 2

Fig 1 shows a low magnification view of a cutaneous nerve fibre in cross section. n nucleus of a Schwann cell, my myelinated and nmy non myelinated axons embedded in the cytoplasm of the Schwann cells, m mitochondria, cf collagen fibers of endoneurial connective tissue. $\times \approx 20000$.
Fig 2 A detail of a myelin sheet showing the pattern of the parallel arranged myelin layers. $\times \approx 150000$.

A⁻ Schwann Cell Cytoplasm

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Fig 5

A portion of the myelinated fibre irradiated by 5800 rep. The axonal membrane (axm) has partially peeled off from the compact myelin (my) forming a loop where a possibly swollen and vacuolated mitochondrial profile of the Schwann cell cytoplasm can be seen & vesicular fragments of the ergastoplasmic channel system
 X ca 76000

axon. The axonal membrane is usually to be seen peeled off from the compact myelin and the space formed in this way is filled with a loose, fine, granular mass, probably *in vivo* fluid, and the axon is thus compressed into a narrow space in the middle of the sheath (Fig 3)

B Nucleus of the Schwann Cell

The normal fine granular and partially filamentar structure of the nucleoplasm is found to have changed even in some of the fibres of the control material incubated in Tasaki Ringer solution. More severe changes in chromaline distribution are regularly observed in the irradiated material apparently representing various stages of nuclear degeneration, which in light microscopy are termed as the hyperchromacy of the nuclear membrane, karyopyknosis, rhexis and lysis. In general, the nuclear membranes are easily distinguished and the pores normally found here are clearly discernible. In the swollen and vacuolised cytoplasm double membranes are seen, originating from the outer nuclear membrane and connecting the perinuclear space to the extracellular room; this was first reported as a normal finding in several cell types by Watson (1955) and Palade (1955). The strong osmiophilic nuclear substance is found as a condensed, rough granular mass closing lining the inner nuclear membrane or as irregular clusters drifting from the nuclear membrane system inside the nuclear space (Fig 6).

In some cases of strongly irradiated material (over 22 000 rep) quite large, distended vacuoles can be noticed in such Schwann cells as have only non myelinated axons embedded in their cytoplasm, or in connection with disrupted myelinated fibres. The distinct double mem

in the Schmidt-Lantermann clefts as well as in the other shearing regions of the myelin sheath (Fig 5). After the smallest doses of irradiation (700–6000 rep), when no fatal destruction of the myelin and the axoplasms occurs, the stretching and tearing of the myelin sheaths in the Schmidt-Lantermann clefts are found to be very striking and constant phenomena (Figs 3 and 4) as compared with the control material. This is connected with a shearing of the adjacent myelin lamellation, where the distance between the two osmiophilic layers might use 800 Å instead of the normal 80–90 Å. However, this swelling in the region of the compact myelin cannot be explained by analogy with the changes described above, for the Schwann cell cytoplasm might have disappeared in the region of the compact myelin (Robertson 1958, Engstrom & Wersall 1958). This kind of swelling of the myelin sheath is described by Wilke (1959) in the nerve fibres of the central nervous system in diabetic coma patients.

Another phenomenon which can be explained in the same way as those described above and which occurs after a small dose of irradiation is the swelling and vacuolisation inside the myelin sheath around the

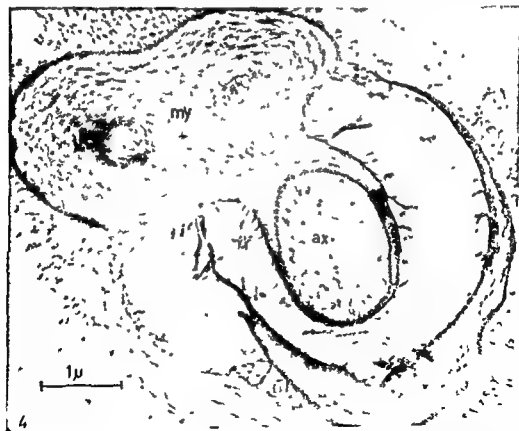


Fig 5

region. A considerable swell
myelin arches in the shearing
Irradiation dose: 5800 rep

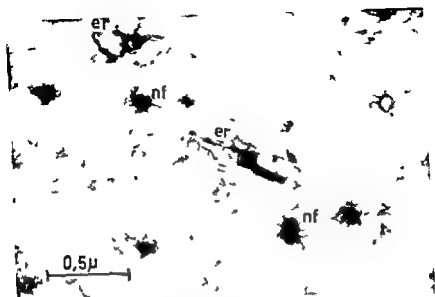


Fig 7

Some details of the axoplasm of a myelinated fibre irradiated by a dose of 5800 rep of clusters of broken neurofilaments accumulating near to the ergastoplasmic fragment on profiles (er) \times ca 44000

Vial (1958) in his study of the changes in the finer structure of peripheral nerves during the Wallerian degeneration also states that the axoplasmic components particularly the ergastoplasmic channel system were the most sensitive to damage in the time course of his experimentation

1 *Neurofilaments* After the smallest dose (750 rep) a fairly well preserved network of neurofilaments can be found in the nerve fibres although elsewhere in the axon Schwann cell system degenerative changes occur (Fig 3) After doses of more than 5000 rep several changes are seen the electron density of the neurofilaments is increased and they appear as thickened broken stumps after doses of more than 20 000 rep usually as rough highly osmiophilic bands or asteroid clusters often lying close to the fragmented vesicles of the ergastoplasmic channel system (Fig 7) In most of the fibres irradiated by more than 30 000 rep the axon cavity is empty or contains some irregular remnants of the axoplasm

2 *Ergastoplasmic channel system* The quickest changes corresponding to the strength of the irradiation dose occur in the ergastoplasmic channel system which in the normal material appears as a series of small clear vesicles

or small clear vesicles often gathering round clusters of broken neurofilaments (Fig 7) In the more irradiated material

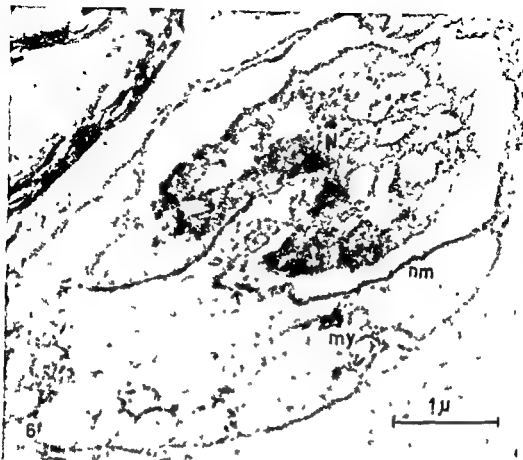


Fig 6

A portion of a Schwann cell irradiated by a dose of 12600 rep. The cytoplasm has lost its structure containing few fine granules and vacuoles, which have double lamellated boundaries connecting them to the cell membranes. These are probably remnants of non myelinated axons embedded in the cytoplasm. In some places myelin spirals around the vacuole can be observed (my). N a clump of nuclear substance nm nuclear membranes, where pores (at the arrows) can be distinguished
 \times ca 28000

brane is seen around the vacuole and is filled with a loose, very fine granular mass, probably *in vivo* fluid. Because of its electron density and the size of the granules this kind of vacuole does not differ from those seen in connection with the vacuolisation of the cytoplasm. Apparently this kind of degeneration product is a karyolytic vacuole (see Fig 11).

C Axoplasmic Components

In the structural components of the axoplasm such severe destruction is observed even after irradiation doses of over 5000 rep, that no intermediate stages in this degeneration process can be distinguished. Methodical artefacts, often found in the unirradiated material incubated in Tasaki-Ringer solution, particularly make interpretation of the slight axoplasmic changes in the experimentation material extremely difficult.

often to be seen in the material irradiated by doses of 2000-20 000 rep. The myelin sheath in these cases torn and the portion of swollen axoplasm has bulged out into the Schwann cell cytoplasm. Remarkable accumulation of spherical bodies is visible in this region. Among these particles most can be identified as mitochondrial elements. They appear to be somewhat swollen and their inner part consists of a quite homogeneous electron dense mass, where the cut surfaces of the internal tubules can be recognized. Between these bodies large clusters of small vesicles, measuring 400-1000 Å in diameter, can often be found. The origin of these mitochondria cannot be explained with certainty, but they probably represent a mixture of axoplasmic and Schwann cell mitochondria. This kind of accumulation of slightly altered mitochondria, seen in connection with the vacuolisation of the cytoplasm, suggests that mitochondria in some way participate in certain degeneration processes of the cell. The possible rôle of the mitochondria in the genesis of some forms of degeneration types of the cytoplasm, e.g. cloudy swelling, has been widely discussed in the recent literature (see *Manuelidis* 1958).

Remnants of seriously altered mitochondria can be found even in largely destroyed axons in the strongly irradiated material (up to 22 800 rep). This is in agreement with the observations of *Vial* (1958), who found the mitochondria to be fairly resistant structures in the Wallerian degeneration.

D Myelin Structures

In the greater part of the irradiated material very many fixation artefacts are to be seen in the myelin sheaths. Therefore, it is impossible to interpret the rôle of irradiation in the pure tearing phenomena of the myelin. In addition to the findings described under paragraph A, some observations of certain changes in the myelin structures of the irradiated material can be made. These findings will form a continuous series of progressive changes corresponding to the strength of the irradiation doses used, thus suggesting that they are vitally produced.

After moderate irradiation (5000-20 000 rep) patterns as illustrated Fig. 10 can be seen, where the destroyed myelin sheath has coiled into secondary windings forming small spiral bodies. In other

h
1
in Fig. 11, are constantly observed. There a central vacuole, probably karyolytic is visible. On the periphery of the formation many vacuoles bounded by irregularly arranged spiral windings, occur. The vacuoles contain a homogeneous, fine, granular mass and sometimes the spiral

especially after doses of more than 20 000 rep the elements in question can no longer be identified

3 The rôle of the granular axoplasmic components in the degeneration process cannot be followed with certainty because of the different destruction products, which are mostly granular in nature and occur predominantly in most of the neurons in the irradiated material

4 *Mitochondria* A moderate swelling and partial fragmentation in the axonal mitochondria can often be seen after a smaller dose of irradiation (700–2000 rep)

Some very impressive patterns reproduced in Figs 8–9 are quite



Fig 8

Fig 8 illustrates a survey on a nerve fibre irradiated by 12600 rep. A myelin sheath (my) is torn and the axoplasm (ax) has partially bulged out into the Schwann cell cytoplasm where a remarkable accumulation of spherical bodies can be seen \times ca 10000

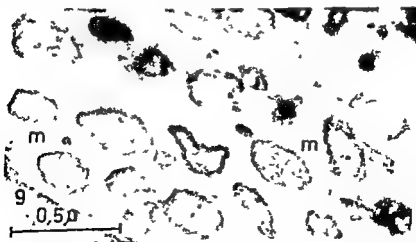


Fig 9

Fig 9 shows a detailed picture of the bodies seen in Fig 8. Most of them can be identified as mitochondrial elements (m) with tubular inner profiles \times ca 48000

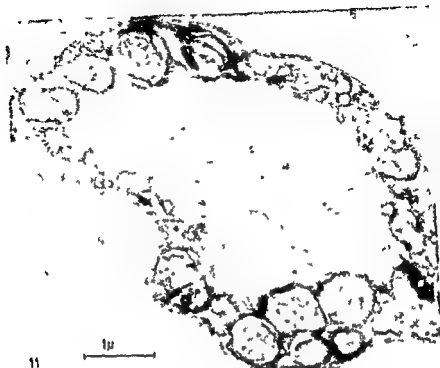


Fig 11

A degenerated portion of a Schwann cell

After irradiation doses of about 5000 rep an almost complete depression of the action potential within 30 minutes of irradiation could be observed. This effect could be obtained within a few minutes of administering doses of more than 10 000 rep. The morphological changes in the fibres after doses of about 5000 rep show striking tearing and a breakdown of the structure of the myelin sheath. The axoplasm appears to be almost completely destroyed and severe damage occurs in the Schwann cells and myelin structures. The question of which elements in the nerve fibre are responsible for the observed changes can only be answered precisely in the light of further studies of the structure of the neuroplasmic channel system. The fragmentation of the ergastoplasmic channel system shows the most clear correlation

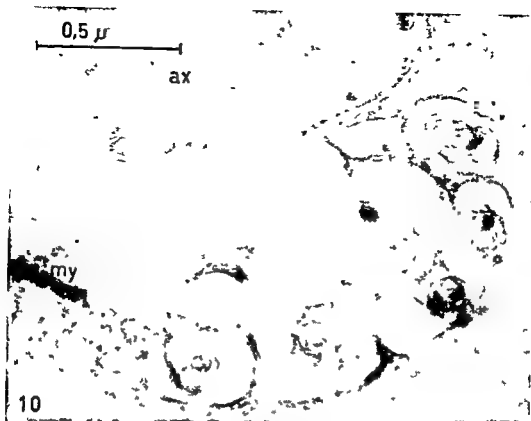


Fig 10

Spiral bodies coiling up from the myelin sheath (my) of a fibre irradiated by 22400 rep at the axonal cavity \times ca 66000

bodies described above are also found in them. These kinds of formations are considered to be degenerated portions of Schwann cells, where only non myelinated axons corresponding to the vacuoles observed are embedded.

In these findings the abundance of irregular myelin coils is striking as compared with the normal material. It is obvious that irradiation causes a secondary, irregular myelination both in degenerated myelin sheaths and in non myelinated axons.

In summing up, when we compared the morphological changes in the nerve fibres irradiated with α particles with the changes in the physiological functions emphasized by Bergstrom, Bläfield & Brenner 1960, it was observed that both the destructive changes in cell structures and the depression of the action potential were highly dependent on the irradiation dose. With the smallest doses used (up to 2000 rep) there was no significant alteration in the action potential during the time of observation (30 minutes). The morphological study of this material chiefly reveals swelling phenomena and degeneration of the nuclei of the Schwann cells. Axoplasmic structures, with the exception of the fragmentation of the ergastoplasmic channel system, were only slightly damaged.

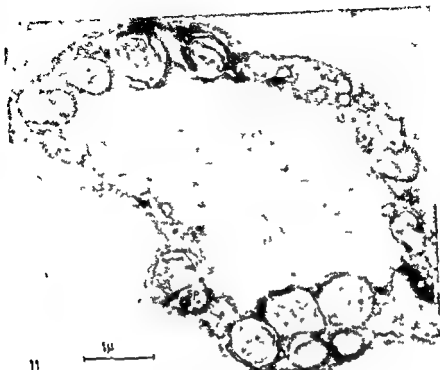


Fig 11

After irradiation doses of about 5000 rep an almost complete depression of the action potential within 30 minutes of irradiation could be observed. This effect could be obtained within a few minutes and the basic structural components of the axoplasm. After bigger doses the axoplasm appears to be almost completely destroyed and severe damage occurs in the Schwann cells and myelin structures. The question of which elements in the nerve fibre are responsible for the answer precisely in the light of the structure of the neurocytoplasmic fragmentation of the ergastoplasmic channel system show the most clear correlation.

SUMMARY

The changes in the finer structure of the sensory nerve fibres of the frog were studied after circumscribed irradiation with Polonium $^{10}\alpha$ particles. After irradiation doses of about 5000 rep an almost complete depression of the action potential could be observed within 30 minutes. The most striking ultrastructural changes in the corresponding material were the fragmentation of the ergastoplasmic channel system of the axoplasm into small vesicles, and the tearing of the neurofilaments. The various forms of degeneration seen after different irradiation doses used in the axon-Schwann cell system are described.

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EVALUATION OF MACRUZ INDEX BASED ON POSTMORTAL ATRIAL MEASUREMENTS

By

ANTTI LISALO, ANSSI TENHUNEN LAURI SAKSA and
OLLI HEINIVAARA

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Increasing attention has been paid in recent times to the shape, axis, and duration of the P wave in the evaluation of electrical activity and in trying to elucidate the pathoanatomical changes in the atria. Macruz and his co-workers (1) introduced a new, simple method by which to assess the enlargement of the atria using the relationship between the duration of the P wave and the duration of the PR segment, i.e. (PR interval—P wave).

The purpose of the present investigation was to compare these ECG and autopsy findings in cases without congenital heart disease and rheumatic valvular disease.

SERIES AND METHODS

The series consisted of 16 patients whose age, sex and cause of death are summarized in Table 1. The ECG was taken within 10 days of death either on a direct writing ECG apparatus or on photosensitive paper at the rate of either 25 mm/sec or 50 mm/sec and in 2 cases at 10 mm/sec. The ECGs were of digitalis produce the

The heart was removed by cutting the vessels in such a way that 15–20 cm of the base of the vessel was left in the heart block. The heart was then washed carefully with water. A short plastic tube was attached to the inferior vena cava and to one of the pulmonary veins and all the other vessels except the aorta and the pulmonary artery were ligated. Water was then run through the heart until all blood had been washed out. The heart was immersed into a dish containing 5 per cent formalin which was run through the heart. Subsequently the aorta and the pulmonary artery were ligated and formalin poured through the veins into the heart thus filling the atria with the solution.

When the heart had hardened for 4 days the formalin was carefully washed out and replaced by plastic resin poured in at standard pressure (Vestopal™ 100 g accelerator 10 g and catalyst 15 g). Care was taken to ensure that the resin filled the atria completely. The hearts were examined on the following day and any deficit of resin was made good. Two days later the hearts were placed in concentrated hydrochloric acid for maceration of the heart muscle. (The method is explained in detail by *Tompsett & Wakeley*).

After maceration the remnants of the cardiac muscle were washed away and the cast was cleaned carefully with a brush. The parts of the heart the blood vessels departing from it and the boundaries between the atria and the ventricles were clearly distinguishable in the cast (Figs 1-3). The actual atrial part could be separated along the boundaries with a saw. The volume of the atrial casts obtained was measured by immersion into water, which was repeated three times.

The values thus obtained may because of the fixation procedure differ slightly from immediate post mortem values and therefore a control study was made in one case immediately after death by filling the atria with chloroform as described above. The volume was measured by letting the chloroform run from the atrio-ventricular border along a thin needle into a graduated flask. Comparison with the cast made from the same heart showed that the chloroform procedure gave values which were about 20 per cent higher. Obviously this was partly due to the impossibility of eliminating the volume of chloroform contained in the vessel stumps which was practicable in the cast measurements. Formalin fixation may be another reason for a reduction in volume. Calculation of the ratio of the volumes from these two experiments gave however practically the same result (1.10-1.09).

TABLE 1

Pat No	Sex	Age years	I n	II n	Index I n II n	Autopsy
1	♂	57	13	102	0.62	Haemorrhagia cerebri
2	♀	59	32	43	0.74	Aneurysma arteriae cerebri mediae Cirrhosis biliaris hepatis Choled cholithiasis
3	♀	42	38	48	0.79	Piel nephritis chronica
4	♀	53	40	34	1.18	Infarctus cordis Cardiosclerosis
5	♀	59	48	55	0.87	Haemorrhagia cerebri Hypertensio arterialis
6	♂	73	78	45	1.73	Lymphosarcoma
7	♂	64	51	71	0.72	Leucemia lymphatica
8	♀	70	50	57	0.88	Piel nephritis chronica
9	♀	60	58	73	0.80	Pielonephritis chronica
10	♀	65	60	75	0.80	Pielonephritis chronica
11	♂	58	76	76	1.0	Carcinoma vesicae urinariae
12	♀	42	50	57	0.89	Haemorrhagia subarachnoidalis Hypertensio arterialis
13	♂	64	98	92	1.07	Carcinoma oesophagi
14	♂	79	54	78	0.69	Carcinoma pulmonis dextri
15	♂	69	64	70	0.91	Carcinoma pancreatis
16	♀	80	86	80	1.07	Haemorrhagia cerebri Hypertensio arterialis

RESULTS

Table 1 shows a series of 16 patients and the post mortem measurements of atrial volumes. Table 2 shows *Macruz* indices calculated from the ICG both with and without enlarger. Cases 8, 12 and 16 had in a block of first degree with a PR interval of 0.23, 0.26 and 0.28. No notable atrial dilatation was demonstrated at autopsy in any of these cases.

DISCUSSION

It appears from Fig. 4 that no distinct correlation prevails between *Macruz* index calculated from the ICG and the atrial volume ratio measured post mortem. When the atrial volume is measured in this

Fig 1

Fig 2



Fig 3

Fig 1 and 2 Dorsal and ventral view of the cast after maceration of the heart muscle

Fig 3 Right and left half of the cast separated after maceration The picture shows the distinct borders of the atria

way it is very possible that the volume is not the same as intra vitam. However it appears that the post mortem changes in either direction in the atria are similar (This is suggested by e.g. our observation in the chloroform measurements in which the obvious shrinkage of the atria failed to change the volume ratio.) Naturally it is impossible at autopsy

After maceration the remnants of the cardiac muscle were washed away and the cast was cleaned carefully with a brush. The parts of the heart, the blood vessels departing from it and the boundaries between the atria and the ventricles were clearly distinguishable in the cast (Figs 1-3). The actual atrial part could be separated along the boundaries with a saw. The volume of the atrial casts obtained was measured by immersion into water, which was repeated three times.

The values thus obtained may because of the fixation procedure differ slightly from immediate post mortem values and therefore a control study was made in one case immediately after death by filling the atria with chloroform as described above. The volume was measured by letting the chloroform run from the atrio-ventricular border along a thin needle into a graduated flask. Comparison with the cast made from the same heart showed that the chloroform procedure gave values which were about 20 per cent higher. Obviously this was partly due to the impossibility of eliminating the volume of chloroform contained in the vessel stumps which was practicable in the cast measurements. Formalin fixation may be another reason for a reduction in volume. Calculation of the ratio of the volumes from these two experiments gave however, practically the same result (1.10-1.08).

TABLE 1

Pat No	Sex	Age years	I n	R n	Index I n R n	Autopsy
1	♂	57	63	102	0.62	Haemorrhagia cerebri
2	♀	59	32	43	0.74	Aneurysma arteriae cerebri mediae Cirrhosis biliaris hepatis Cholecholelithiasis
3	♀	42	38	48	0.79	Pyelonephritis chronica
4	♀	53	40	34	1.18	Infarctus cordis Cardiosclerosis
5	♀	59	48	55	0.87	Haemorrhagia cerebri Hypertensio arterialis
6	♂	73	78	45	1.73	Lymphosarcoma
7	♂	64	51	71	0.72	Leucaemia lymphatica
8	♀	70	50	57	0.88	Pyelonephritis chronica
9	♀	60	58	73	0.80	Pyelonephritis chronica
10	♀	65	60	75	0.80	Pyelonephritis chronica
11	♂	58	76	76	1.0	Carcinoma vesiculae urinariae
12	♀	42	50	57	0.89	Haemorrhagia subarachnoidalis Hypertensio arterialis
13	♂	64	98	92	1.07	Carcinoma oesophagi
14	♂	79	54	78	0.69	Carcinoma pulmonis dextri
15	♂	69	64	70	0.91	Carcinoma pancreatis
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RESULTS

Table 1 shows a series of 16 patients and the post mortem measurements of atrial volumes. Table 2 shows *Macruz* indices calculated from the ECG both with and without enlargement. Cases 8, 12 and 16 had an a-v block of first degree with a PR interval of 0.23, 0.26 and 0.28. No notable atrial dilatation was demonstrated at autopsy in any of these cases.

DISCUSSION

It appears from Fig. 4 that no distinct correlation prevails between *Macruz*' index calculated from the ECG and the atrial volume ratio measured post mortem. When the atrial volume is measured in this

in ECG and vectorcardiography in conditions involving systolic or diastolic overloading of the atria have given contradictory results (Scheuer *et al*). This is why cases of congenital heart disease or acquired valvular disease were excluded from the present series—they involve many and special hemodynamic conditions which are even more difficult to compare with autopsy findings. It might be permissible to conclude perhaps that even if the values of atrial volume are not absolute in the present series they are comparable. On the other hand, the electrolytic disturbances known to occur in chronic infections of the urinary tract and in malignant diseases of long duration caused hardly any conduction disturbances in the present series. The extracellular electrolytic values were normal in all cases in which a negative autopsy Macruz' index correlation was established (2, 3, 9, 15).

Considerable differences have been established also in the normal distribution of the duration of the P wave (Caceres & Kelsor). If it be assumed that the electrical activity of the atria is best indicated by the P wave the wave of longest duration, the frequently proposed normal values for the P wave may have been too short. The determination of P without an enlarger may be one of the factors. Table 2 shows the values for both P and PR segment determined with and without an enlarger. It is seen readily from the table how often differences, small though they are, are present in the calculation of the duration of the P wave and the PR segment. The differences caused no decisive changes in the index in the present series, but sometimes even a small fluctuation in the duration of the P wave or the PR segment may be of decisive significance if the normal limits of the index are limited to the 1.0-1.6 range. The greatest possible accuracy is a natural requirement of the measurements but this point deserves emphasis also because the use of an enlarger is probably not yet fully established.

In addition to the absence of correlation between Macruz' index and the autopsy findings, another special feature was the pronounced deviation of the index values. Kahn and his co-workers established abnormally high and low index values in coronary disease, attributing these to conduction disturbances caused by coronary insufficiency in the atrial area and the subsequent changes in the shape and duration of the P wave. A corresponding factor might have caused the marked deviation of the indices in the present series, considering the high age of the patients and the high incidence of coronary sclerosis in these age groups. This is a mere hypothesis since unfortunately the volume measurements made it impossible to study the coronary vessels. Study of the coronary circulation should perhaps have been focused on the atrial region although the role of local lesions in the atrial region in the duration of the P wave cannot as yet be stated with any certainty (Abildskov).

Granting the not fully established assumption that the ratios of volume determined at autopsy are true, it can be said that the series

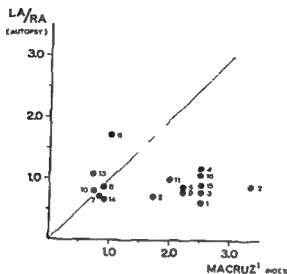


Fig. 5

Diagram showing the relation between Macruz index and postmortal atrial measurements (left atrium/right atrium)

to duplicate perfectly the hemodynamic conditions *intra vitam* and this may be one reason why data on the normal volume of the atria vary so greatly (*e.g.* Hochrein, Boyd). The differences in hemodynamics in the atrial area may cause changes, occasionally even considerable changes, in the electrical activity of the atria and thus in the shape and duration of the P wave. Our knowledge of the effect of these factors (systolic and diastolic overloading) is still very incomplete and it is very difficult to distinguish between hypertrophy and dilatation *intra vitam*. In fact this is a problem even at autopsy. Studies of the P wave

TABLE 2

P-PR Segment and Macruz Index determined with or without the Enlarger

Pat. No.	With enlarger			Without enlarger		
	P	1st segm	Index	P	PR segm	Index
1	0.10	0.04	2.5	0.09	0.04	2.3
2	0.10	0.06	1.7	0.08	0.03	1.0
3	0.10	0.04	2.5	0.03	0.04	2.0
4	0.10	0.04	2.5	0.09	0.04	2.3
5	0.11	0.05	2.2	0.10	0.04	2.5
6	0.08	0.03	1.0	0.07	0.08	0.9
7	0.07	0.09	0.8	0.07	0.09	0.8
8	0.11	0.12	0.9	0.11	0.12	0.9
9	0.11	0.05	2.2	0.10	0.04	2.5
10	0.10	0.14	0.7	0.09	0.13	0.7
11	0.08	0.04	2.0	0.08	0.03	2.7
12	0.10	0.03	3.3	0.10	0.03	3.3
13	0.11	0.15	0.7	0.10	0.17	0.7
14	0.10	0.11	0.9	0.10	0.11	0.9
15	0.10	0.10	2.5	0.09	0.04	2.3
III	0.20	0.08	2.5	0.19	0.03	2.0

THE EFFECT OF A PHENOL WATER SOLUBLE FRACTION OF THE TUBERCLE BACTERIUM UPON THE SKIN (IN VIVO) AND MIGRATING LEUKOCYTES (IN VITRO) OF TUBERCULIN SENSITIZED GUINEA PIGS¹

By

TH. PACKALÉN, J. WASSERMAN and C. WEIBULL

Received 3 JULY 61

In our earlier experiments (12, 13, 15, 16) we have shown that various bacterial lipopolysaccharides of endotoxin nature have *in vitro* a cytotoxic effect on cells from tuberculin sensitized guinea pigs. Since it is generally assumed that the tuberculin reaction *in vivo* is elicited by specific tuberculo-proteins this non-specific hypersensitivity prompts the question whether the hypersensitivity phenomena *in vivo* and *in vitro* have at all a basic mechanism in common. According to O'Neill & Favour (11) the tuberculin cytotoxicity *in vitro* might be that portion of the tuberculin reaction *in vivo* which is specific and which triggers off complex non-specific inflammatory reactions. Lasfargues and coworkers (7) on the other hand have suggested that the cellular hypersensitivity phenomena *in vitro* might be due merely to a lowering of the vitality of the tissues by the pathological process.

When it was demonstrated that nonprotein substances of non-specific bacteria exert a cytotoxic effect on tuberculin sensitized tissues *in vitro*, it was felt to be highly desirable to study the cytotoxicity of nonprotein fractions of the sensitizing tubercle bacilli themselves. It proved difficult to effect a complete removal of the protein moiety from an extract of tubercle bacteria when no degradation of the remaining nonprotein constituents occurred.

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tubercle bacteria. The protein content remained too high even when the phenol extraction procedure was repeated. Instead, the initial material chosen was a preparation obtained by freezing and thawing living tubercle bacteria according to the method of Billaudelle & Warfvinge (2).

¹ This investigation was supported by grants from the Swedish National Association against Heart and Chest Diseases.

included no cases with distinct dilatation of the atria. The selection of the material indicates the same. Hence the possible factors should be considered which might have caused what the authors assume to be "incorrect" index values.

The series may give certain indication in this respect. In some cases the deviation of the values given in the illustration showed a distinct correlation (7, 8, 10, 13, 14), and in others again a clear lack of correlation (1, 3, 4, 5, 9, 12, 15, 16). The clinical data and the autopsy findings showed that hypertrophy of the left ventricle was diagnosed in the majority of the cases in the latter group and that *e.g.* all cases of cerebral hemorrhage belonged to this group. The data are insufficient for the establishment of the possible role of coronary sclerosis, principally because it was not possible at autopsy to examine the blood vessels. A more thorough comparison of this kind would require a considerably larger series of patients.

SUMMARY

The correlation between *Macruz'* index calculated from the ECG and the atrial volume measured at autopsy was studied in 16 cases. No distinct correlation was established. There was pronounced deviation in the index values although the atrial volume values established at autopsy did not rise to a pathological level. The difficulty of comparing electrical activity and autopsy values is discussed, and also the factors which might contribute to the deviation of *Macruz'* indices established in the series.

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SKIN (IN VIVO) AND MIGRATING LEUKOCYTES
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Received 3 viii 61

In our earlier experiments (12, 13, 15, 16) we have shown that various bacterial lipopolysaccharides of endotoxin nature have *in vitro* a cytotoxic effect on cells from tuberculin-sensitized guinea pigs. Since it is generally assumed that the tuberculin reaction *in vivo* is elicited by specific tuberculoproteins this nonspecific hypersensitivity prompts the question whether the hypersensitivity phenomena *in vivo* and *in vitro* have at all a basic mechanism in common. According to O'Neill & Favour (11), the tuberculin cytotoxicity *in vitro* might be that portion of the tuberculin reaction *in vivo* which is specific and which triggers off complex nonspecific inflammatory reactions. Lasfargues and coworkers (7) on the other hand, have suggested that the cellular hypersensitivity phenomena *in vitro* might be due merely to a lowering of the vitality of the tissues by the pathological process.

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fractions of the sensitizing tubercle bacilli themselves. It proved difficult to effect a complete removal of the protein moiety from an extract of tubercle bacteria when no degradation of the remaining nonprotein constituents was desired. The water-soluble lipopolysaccharides from tubercle bacteria (17) did not
tubercle bacteria. The protein content remained too high even when the phenol extraction procedure was repeated. Instead, the initial material chosen was a preparation obtained by freezing and thawing living tubercle bacteria according to the method of Billaudelle & Warfvinge (2).

¹ This investigation was supported by grants from the Swedish National Association against Heart and Chest Diseases.

This preparation, named Endotuberculin by the authors, is reported to consist chiefly of protein lipopolysaccharide complexes (1). Removal of the majority of the protein moiety was achieved by subsequent phenol water extraction. The present paper deals with the effect of this phenol extracted fraction upon the skin (*in vivo*) and the leukocytes (*in vitro*) of tuberculin-sensitized guinea pigs.

MATERIALS AND METHODS

Animals

Male guinea pigs weighing 400-600 gm were sensitized by BCG vaccination and injections of killed bovine tubercle bacilli emulsified in paraffin oil (16). The tuberculin sensitivity of the animals was tested by an intracutaneous injection of 0.1 ml of standard Old Tuberculin (OT) diluted 1:100. Untreated male guinea pigs weighing 400-600 gm and reacting negatively to OT were used as controls.

Preparations

Old Tuberculin. A batch of tuberculin (strength 11,200 IU per 0.1 ml) prepared from a H37Rv strain and without preservatives was used. It was employed in a dilution of 1:100.

Endotuberculin (ET). Lot No. 4 of IT¹ extracted from living tubercle bacilli H37Rv grown in Sautons' broth according to the method described by Billaudelle & Warfvinge was used (2). The dry weight of the preparation was 1.9 per cent determined after drying samples at 100° for 24 hours, and the protein content was 23 per cent of the dry weight. The protein content was estimated by the Folin Wu method (9) after preceding precipitation with 10 per cent trichloroacetic acid at room temperature.

Phenol extract of Endotuberculin (PFT). One part of lot No. 4 was extracted by a phenol water mixture at 65°C according to the procedure described by Westphal and associates (17) then cooled and centrifuged. The supernatant was dialyzed for 6 days against running tap water and then for 2 days against distilled water. The dry weight of the preparation was 0.28 per cent and the protein content 0.4 per cent of the dry substance. The latter was determined after precipitation with trichloroacetic acid by the method of Folin and Wu (8). By the same method the amount of peptides not precipitable with TCA was 2.3 per cent. Fig. 1 represents a paper chromatogram of PFT hydrolyzed in 2N HCl for 2 hours at 100°C. The chromatogram shows the preparation together with an early prepared batch of PFT containing 1.2 per cent. The nitrogen determination method used for the lipid determination was the following. The PFT was hydrolyzed with 6N HCl for 2 hours at 100°.

The acid solution was repeatedly extracted with ether and the ether extract was treated with a stream of carbon dioxide at room temperature. The residue was dried *in vacuo* at room temperature and redissolved in ether. The solution now obtained was evaporated as described above and the residue was weighed after drying *in vacuo*.

The Method of Experimentation

For skin testing the preparations were generally used in tenfold dilutions. For *in vitro* experiments two fold dilutions were employed starting from the maximal dose that did not appreciably inhibit the migration of leukocytes from normal animals. The amounts of the preparations used in each experiment are expressed as the weight of dry substance. The capacity of ET and PFT to produce delayed type skin reactions was studied in normal and tuberculin sensitized guinea pigs. Reactions were read 48 hours after challenge.

1. Kindly made available by Dr I. Silfverstolpe, the State Bacteriological Laboratory, Stockholm.

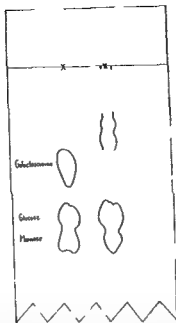


Fig. 1

Right: Paper chromatogram of phenol treated and hydrolyzed (2 hrs. in 2N HCl at 100°C) Endo tuberculin; Left: Chromatogram of a mixture of mannose, glucose and galactosamine. Solvent: ethyl acetate:acetic acid:water (3:1:1). Developed: silver nitrate:alcohol:potassium hydroxide.

The determination of the cytotoxic effect of the two preparations was based on measurements of inhibition of leukocytic migration in the presence and absence of these substances (16). In some experiments the migration of leukocytes was also tested in the presence of BCF tuberculin.

RESULTS

Skin Reactions

320 mcg of IT was injurious to the skin of both normal and sensitized animals. An intracutaneous dose of 32 or 3.2 mcg of FT did not cause any skin reaction in normal animals. When injected into the skin of tuberculin sensitized guinea pigs both doses elicited delayed skin reactions of tuberculin type. No skin reaction followed the injection of 0.32 mcg. The smallest amount of FT still displaying skin activity in tuberculin sensitized animals was 0.64 mcg. 340 mcg of PET was

activity (Table 1). 1 mcg of PET were practically devoid of any skin

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Endotuberculin (ET) Lot No. 4 of LT1 extracted from living tubercle bacilli H37Rv grown in Sauton's broth, according to the method described by Billaudelle & Warfvinge was used (2). The dry weight of the preparation was 1.9 per cent, determined after drying samples at 100° for 24 hours, and the protein content was 2.3 per cent of the dry weight. The protein content was estimated by the Lohm-Wu method (9) after preceding precipitation with 10 per cent trichloroacetic acid at room temperature.

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1. Kindly made available by Dr I. Silverstolpe, the State Bacteriological Laboratory, Stockholm.

TABLE 2

Cytotoxic Indices of ET, PET and Old Tuberculin for Migration of Leukocytes from Normal and Tuberculin Sensitized Guinea Pigs

Animal	Date 1959	Control Migration (mm)	ET mcg/ml		PFT mcg/ml				CIT 1/100
			32	16	25	11	7	35	
Normal Animals									
504	1 9	1 50	1 15		0 76				1 07
505	1 9	1 77	1 11		1 00				0 64
529	1 23	1 78	1 01		0 65				0 98
532	1-23	2 16	0 89		0 94				0 94
502	1 27	1 71	0 86		0 84				0 98
503	1-28	1 62	1 32						0 95
514	2 2	1 65	0 98		0 93				1 04
516	2 2	2 11	1 04		1 05				
513	3 31	2 74			1 01				
519	3 31	1 70			1 00				
529	3 31	1 63			0 92				
528	4-2	1 56			1 13				
502	4 3	1 87			1 09				
503	4-3	2 32			1 01				
504	4-3	1 27			1 11				
505	4 3	1 93			1 04				
Sensitized Animals									
555	2 2	1 78	0 41		0 20				0 55
556	2 2	1 51	0 43		0 40				0 37
579	2 21	1 28	1 04		0 87				0 32
581	2 21	2 13	0 46		0 40				0 40
570	2-23	1 14	0 46		0 53				0 37
571	2 23	1 67	0 46		0 51				0 41
594	2 24	1 80	0 35		0 32				0 29
596	2 24	2 50	0 45		0 34				0 43
601	2 24	1 21	0 48		0 46				0 44
572	2-25	1 47	0 50	0 61	0 48	1 00			0 50
573	2 25	1 56	0 43	0 60	0 42	0 60			0 56
586	3 4	1 77	0 32	0 40		0 38	0 47		
569	3 5	2 48	0 44	0 52		0 36	0 54		
580	3-7	1 58	0 40	0 61		0 35	0 51		
582	3 7	1 43	0 38	0 53		0 41	0 40		
570	3 9	1 91		0 48			0 44	0 77	
571	3 9	1 49		0 74			0 53	0 68	
593	3-10	1 93		0 39			0 73	0 44	
591	3 12	2 30		0 70			0 61	0 73	
586	3 13	1 43		0 70			0 48	0 90	
589	3-13	1 71		1 10			1 05	1 09	
568	3-14	2 04		0 60			0 39	0 51	
571	3 16	2 21		0 40			0 33	0 52	
573	3 16	2 41		0 66			0 64	0 65	
571	4 6	2 18					0 32	0 47	0 44
572	4-6	2 21					0 58	0 67	0 72
573	4 6	1 63					0 67	0 73	0 90

TABLE 1

Diameters (mm) of Delayed Type Cutaneous Reactions in Tuberculin Sensitized and Normal Guinea Pigs Challenged with FT and P&T

Animal	Mg of E1 injected					Mg of P&T injected		
	320	32	3.2	0.61	0.12	310	31	3.1
<i>Normal Animals</i>								
497	—*	—				—	—	
498	—	—				—	—	
499	3×6	—				—	—	
500	3×7	—				—	—	
501	—	—				3×4	—	
522	—	—				—	—	
523	—	—				2×3	—	
524	—	—				2×3	—	
525	5×5	—				—	—	
526	—	—				—	—	
567			—	—			—	
568			—	—			—	
569			—	—			—	
571			—	—			—	
572			—	—			—	
<i>Sensitized Animals</i>								
557	10×16	11×12				10×12	12×15	
558	10×15	10×15				? **	?	
559	15×15	12×15				10×15	7×7	
561		20×20					20×20	8×8
564		15×15	?				8×9	—
565		20×20	5×5			10×10	15×15	—
566		15×15				15×15		—
567		14×14	2×4			12×14	—	—
568		20×20	15×15				18×20	—
581		12×14	10×12				10×12	
587		11×17	5×10				15×20	
591		12×14	10×11				7×10	
594		15×16	10×10				15×15	
597	10×10	10×10				—	—	
598	15×15	10×11				5×5	4×5	
599	17×17	12×12				7×7		
600	17×20	7×7				10×10		
601	10×12	10×10				7×10		
602			16×20	10×12	—		11×15	
603			18×20	12×16	—		15×22	—
604			10×16	5×10	—		20×25	—
605			25×25	11×17			20×20	—
606			15×17	7×10			25×30	—
607			19×20	13×15			20×25	
608			20×25	12×17			20×23	
609			17×17	10×13	—		17×25	
610			18×20	7×10			17×17	
611			15×20	7×10			20×22	

* no reaction

** doubtful reaction

TABLE 2

Cytotoxic Indices of ET PFT and Old Tuberculin for Migration of Leukocytes from Normal and Tuberculin Sensitized Guinea Pigs

Animal	Date 1958	Control Migration (mm)	FT mcg/ml		PFT mcg/ml				OT 1:100
			32	16	28	14	7	3.5	
Normal Animals									
504	1 9	1 50	1 15		0 76				1 07
505	1 9	1 77	1 11		1 00				0 64
529	1-23	1 78	1 01		0 65				0 98
532	1 23	2 16	0 89		0 94				0 94
502	1 27	1 71	0 86		0 84				0 98
505	1-28	1 62	1 32						0 95
514	2 2	1 65	0 98		0 93				1 04
516	2 2	2 11	1 04		1 05				
513	3 31	2 74			1 01				
519	3 31	1 70			1 00				
529	3-31	1 63			0 92				
528	4-2	1 56			1 13				
502	4 3	1 87			1 09				
503	4 3	2 32			1 01				
504	4-3	1 27			1 11				
505	4 3	1 95			1 04				
Sensitized Animals									
555	2 2	1 78	0 41		0 20				0 55
556	2 2	1 51	0 48		0 40				0 37
579	2-21	1 28	1 04		0 87				0 32
581	2 21	2 13	0 46		0 40				0 40
570	2 23	1 14	0 46		0 55				0 37
571	2-23	1 67	0 46		0 51				0 41
594	2 24	1 80	0 35		0 33				0 29
596	2 24	2 50	0 45		0 34				0 43
601	2 24	1 21	0 48		0 46				0 44
572	2 25	1 47	0 50	0 61	0 48	1 00			0 50
573	2 25	1 56	0 43	0 60	0 42	0 60			0 56
586	3 4	1 77	0 32	0 40		0 38	0 47		
569	3 5	2 48	0 44	0 52		0 36	0 54		
580	3 7	1 58	0 40	0 61		0 35	0 51		
582	3 7	1 43	0 38	0 53		0 41	0 40		
570	3 9	1 91		0 58					
571	3 9	1 49		0 74			0 44	0 77	
595	3 10	1 93		0 39			0 53	0 68	
591	3 12	2 30		0 70			0 33	0 44	
586	3-13	1 45		0 70			0 61	0 73	
589	3 13	1 71		1 10			0 48	0 90	
568	3-14	2 04		0 60			1 05	1 09	
571	3 16	2 24		0 40			0 39	0 51	
573	3 16	2 41		0 66			0 38	0 52	
571	4 6	2 18					0 64	0 65	
572	4-6	2 21				0 32	0 47	0 44	
573	4 6	1 63				0 58	0 67	0 72	
						0 67	0 73	0 90	

Leukocyte Migration Inhibition

The experimental data presented in Tables 2 and 3 demonstrate the cytotoxic effect of LT, PLT and OT on leukocytes from tuberculin sensitive animals. The smallest amounts still exhibiting cytotoxic activity were 16 mcg per ml for LT and 3.5 mcg per ml for PLT, the respective concentrations of protein being 3.7 mcg per ml for LT and 0.014 mcg per ml for PLT. The content of peptides for PLT was about 0.08 mcg per ml.

TABLE 3
Probability Values for the Cytotoxic Effect of LT, PLT and Old Tuberculin on the Migration of Leukocytes

Preparation	Amount	N_1	N_2	\bar{X}_1	\bar{X}_2	t	P
	mcg/ml						
LT	32	8	15	1.05	0.47	8.29	<0.001
	16		15		0.61	6.03*	<0.001
PLT	28	15	11	0.97	0.45	8.79	<0.001
	14		9		0.52	6.30*	<0.001
	7		16		0.54	7.65*	<0.001
	3.5		12		0.70	4.21*	<0.001
	Concentration						
Old Tuberculin	1:100	7	11	0.94	0.42	9.72	<0.001

N_1 = number of experiments with leukocytes from normal animals

N_2 = number of experiments with leukocytes from tuberculin sensitized animals

\bar{X}_1 = mean cytotoxic index for experiments with leukocytes from normal animals

\bar{X}_2 = mean cytotoxic index for experiments with leukocytes from tuberculin sensitized animals

Probability values calculated according to Student's t test

$$t = (\bar{X}_1 - \bar{X}_2) \sqrt{\frac{(N_1 + N_2 - 2) \bar{S}_1 \bar{S}_2}{N_1 + N_2 - 5 (\bar{X}_1 - \bar{X}_2)^2 + 5 (\bar{S}_1 - \bar{S}_2)^2}}$$

* t values are obtained by comparing the mean cytotoxic indices for the several concentrations of the preparation tested on sensitized leukocytes with the mean cytotoxic index for the single concentration tested on normal cells

DISCUSSION

When discussing the implications of the above mentioned results, the relative amounts of protein and nonprotein components of the tuberculin and PLT and LT preparations, respectively, should be kept in mind.

The smallest doses which elicited typical delayed skin reactions in tuberculin-sensitized guinea pigs were about 0.64 mcg of LT and about 34 mcg of PLT, corresponding to a protein content of 0.15 mcg for LT and 0.14 mcg for PLT, and to a content of 0.49 and 33.86 mcg respectively, of nonproteinic material. The threshold of skin activity seems thus to be related to the protein content and not to the total quantity

of substance injected, nor to the nonprotein part of it. Furthermore, the above mentioned protein quantities are of the same order of magnitude as the smallest amounts of PPD—about 0.5 mcg—reported as still eliciting tuberculin reactions in sensitized guinea pigs (5). These observations prompt the assumption that the skin activity of ET and PET comes from their tuberculoprotein component.

The ET preparation inhibits the migration of leukocytes *in vitro* down to a dilution containing 3.7 mcg of protein per ml. A review of literature shows that the lowest concentrations of tuberculoprotein demonstrated to have cytotoxic activity have been about 1.0 mcg per ml (4, 5, 14). The cytotoxic activity of the ET preparation, may thus, well be attributed to its protein moiety, provided at least a part of it is specific tuberculoprotein. The protein content of PET in the concentration of 3.5 mcg per ml, which was the smallest dose still having a cytotoxic effect, is about 0.014 mcg per ml, which is far below the afore-mentioned minimum amount, of tuberculoprotein. It seems likely that the nonprotein fraction of PET possesses cytotoxic activity *in vitro*. Whether the nonprotein substances are responsible for all the activity of the untreated ET-preparation, or whether there is a combined action of tuberculoprotein and nonprotein material cannot be answered at present. It should not be forgotten in this connection that even purified tuberculoprotein preparations contain appreciable amounts of nonprotein material (8). The possibility thus also exists that their *in vitro* activity may be due, at least in part, to nonprotein substances.

Chemical analysis showed that the nonprotein part of PET contained peptides, lipides and carbohydrates. As the preparation was the nondialysable part of the phenol-water extract, the carbohydrates must be rather high-molecular-weight polysaccharides, some of them probably bound to the lipides present in the extract and constituting lipopolysaccharides. In any case, lipopolysaccharides have been demonstrated in culture filtrates of tubercle bacteria (6) and the method of preparing ET, repeated freezing and thawing, in all probability liberates the constituents of tubercle bacteria in similar way as the spontaneous autolysis occurring in the culture medium during growth. As PET was produced by the subsequent treatment of the ET-preparation with phenol-water, a method used for the extraction of lipopolysaccharides, a portion of the lipopolysaccharides presumably present in ET should also have been included in the PET fraction.

It is difficult to decide with certainty which nonprotein component of PET is active *in vitro*. A group of Japanese investigators have reported on a dialysable peptidic component of tubercle bacilli which possesses strong tuberculin activity (10, 18). Since PET contained only nondialysable peptides, the active part of PET cannot be identical with this factor. The amount of peptides in the smallest dose of PET still active *in vitro* was about 0.08 mcg per ml, which is only about one tenth of the smallest dose of tuberculoprotein known to be cytotoxic *in vitro*,

but by no means too small an amount to have an activity *per se*. On the other hand polysaccharides and lipopolysaccharides represent the bulk of the preparation and may well be responsible for its cytotoxic activity. It should be mentioned, however, that Fabrizio (3) using culture filtrate lipopolysaccharides (6) failed to show any cytotoxic effect on leukocytes from tuberculous guinea pigs (NB in a rather limited series of experiments).

Whether the cytotoxic activity of PET *in vitro*, demonstrated in the present experiments, is specific in an immunological sense, or is a phenomenon to be compared with the various nonspecific reactions elicited by the endotoxin lipopolysaccharides from gram negative bacilli, cannot be determined from the present set of data. The results do suggest, however, that different constituents of PET might be responsible for its effect upon tuberculin sensitized tissue *in vivo* and *in vitro*, respectively. If this holds true, the basic mechanisms of the hypersensitivity phenomena *in vivo* and *in vitro* may also be different. Until this question is definitely settled care should be exercised when drawing parallels between *in vitro* findings and *in vivo* phenomena in tuberculin hypersensitivity.

SUMMARY

Repeated freezing and thawing of tubercle bacilli followed by phenol-water treatment of the extract and dialysis yielded a preparation, the major part of which consisted of polysaccharides. Its minor constituents were nondialysable peptides (2.3 per cent) lipides (1-2 per cent) and proteins (0.4 per cent). This preparation was tested for activity on tuberculin sensitized tissues. *In vivo*, a delayed type skin reaction was elicited in sensitized guinea pigs, only when the content of protein impurity in the dilution tested was at least of an order of magnitude equivalent to the smallest dose of tuberculoprotein known to produce a definite tuberculin reaction. *In vitro*, an inhibiting effect on the migration of leukocytes from tuberculin-sensitized guinea pigs was still displayed by dilutions, the protein content of which was far below the smallest amount of tuberculoprotein still shown to be active *in vitro*. It is concluded that some constituent or constituents of the nonprotein fraction of the tubercle bacterium possess a cytotoxic effect on tuberculin-sensitized leukocytes *in vitro*.

The implications of these results are discussed.

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CORRELATION BETWEEN STAPHYLOCOCCAL PHAGE GROUPS AND SOME STAPHYLOCOCCAL ENZYMES DEMONSTRATED BY SIMPLE METHODS

By

TH. M. VOGLIANG, A. WORMNES and B. ØSTBYGJØD

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Enzyme production is a very common phenomenon of microbes. Demonstration of certain enzymes may be of importance, not only in the differentiation of bacterial species, but also in the separation of pathogenic from non-pathogenic bacterial strains. Thus, coagulase production is the most common criterion used to distinguish pathogenic from non-pathogenic staphylococci. The staphylococci, however, also produce several other enzymes, the determination of which may be of interest, especially if they are of importance to the pathogenicity.

Almost all staphylococcal strains produce hyaluronidase and the question of whether there is any relationship between the quantitative hyaluronidase production and the clinical picture of a staphylococcal infection has arisen. While some investigators have been unable to demonstrate such a connection (11, 13), *Faber & Rosendal* (2) have observed a certain correlation between phage type 71 and strains belonging to group III and their quantitative production of hyaluronidase. With regard to the notorious phage pattern 80/81, however, they found that it produced only moderate amounts of hyaluronidase.

Hyaluronidase may be demonstrated by several physico-chemical and biological methods. At present most of these reactions are so time-consuming that they are unsuitable for routine examination of large series. It is, therefore, important that we now have some simple methods of demonstrating this and also other staphylococcal enzymes. In this communication we have used very easy methods to demonstrate the presence of different enzymes produced by the pathogenic staphylococci, *viz.* hyaluronidase, phosphatase, fibrinolysin and lipase, and compared them to the phage grouping.

MATERIAL AND METHODS

The total series consists of 1738 coagulase positive strains which were isolated from the routine material sent to the laboratory for bacteriological examination during 1959.

Hyaluronidase. Murray & Pearce's (8) decapsulation test was used for the demon-

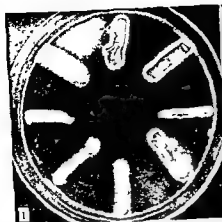


Fig 1 Hyaluronidase



Fig 2 Phosphatase

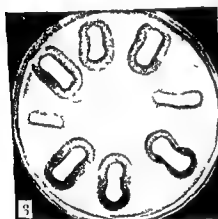


Fig 3 Fibrinolysin

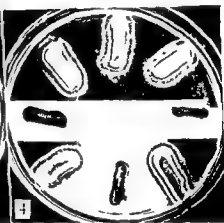


Fig 4 Lipase

Spot inoculation of six coagulase positive and two coagulase negative staphylococcal strains on each Petri dish

stration of hyaluronidase. These authors used a mucoid streptococcal strain as test organism. However, such strains have a tendency to lose their mucoid character on repeated transfer. We have therefore used a *Bacterium anitratum* strain instead, as recommended by Oehring (9). This strain was inoculated on ordinary blood agar plates followed by spot inoculation on the same medium of the staphylococcal strains under examination. The results were read after incubation at 37° C for 18-22 hours. Around the staphylococcal strains which produce hyaluronidase, the *Bact. anitratum* grows with non-mucoid colonies (Fig 1).

Phosphatase. Phenolphthalein phosphate, as recommended by King (7), was used for the determination of phosphatase. A 0.01 per cent solution of phenolphthalein phosphate in water was added to the medium. After held over an open bottle of ammonia, the medium turned red (Fig 2).

Fibrinolysin. For the demonstration of fibrinolysin, 12 per cent human citrated plasma was added to nutrient agar. After standing in a water bath at 56° C. for 15

minutes the mixture was poured into Petri dishes. The production of fibrinolysin reduces the turbidity of the medium resulting—after incubation at 37° C for 18-22 hours—in a clear zone around the staphylococcal colonies (Fig. 3).

Lipase. This enzyme was demonstrated by spot inoculation of staphylococci on nutrient agar containing Tween 80 and CaCl₂ as recommended by Sierra (12). The lipase splits the free fatty acids which combine with the calcium salt and precipitate as fine crystals around the staphylococcal colonies (Fig. 4).

Bacteriophage typing. The phages used are the basic set obtained from the Staphylococcal Reference Laboratory, Colindale. The basic series is composed of 20 phages which are divided into groups (Table 1). We have excluded type 73 in group III that was used in our earlier examinations (3, 14-16). On the other hand some unclassified additional phages have been used. As phages 81, 82 and K56 seem to be related to phages 52, 52A and especially to phage 80 they are included in group I which therefore consists of 8 phages.

TABLE 1
Phages Belonging to the Different Phage Groups

Phage group	Phages in the group
I	29, 52, 52A, 79, 80, 81, 82, K56
II	3A, 3B, 3C, 55, 71
III	6, 7, 42I, 47, 53, 54, 75, 77, 83
IV	42D
V	187

TABLE 2
Enzyme Production of the Staphylococcal Strains

Enzyme		Coagulase positive strains	Coagulase negative strains
Hyaluronidase	positive	1738 (100 %)	0
	negative	0	100 (100 %)
Phosphatase	positive	1738 (100 %)	25 (25 %)
	negative	0	75 (75 %)
Fibrinolysin	positive	1670 (96.1 %)	0
	negative	68 (3.9 %)	100 (100 %)
Lipase	positive	1227 (70.6 %)	11 (11 %)
	negative	511 (29.4 %)	89 (89 %)

TABLE 3
Distribution of the Coagulase positive Strains with Regard to Origin of Samples

Origin	Total material	Lipase negative strains		Fibrinolysin negative strains	
		No.	Per cent	No.	Per cent
Pus	811	234	28.9	27	3.3
Sputa	266	95	35.7	13	4.5
Urine	146	48	32.9	5	3.4
Nose and/or Throat Swabs	485	121	25.6	20	4.1
Blood Cultures	30	10		1	
Grand Total	1738	511	29.4	68	3.9

Phage 83 is included in group III and phage 187 which mainly gives lysis without other phages has been placed in a new group V. Some of the phage patterns may overlap from one group to another especially group I and III and the strains which

with phages
with the same
d phages are

recorded as non typable (NT)

TABLE 4

Distribution of the Coagulase Positive Strains According to Negative Reactions and Phage Groups

Material		Phage groups						
		I	II	III	IV	V	Unc	NT
Coagulase pos strains	No 1738	774	118	479	2	51	184	130
	Per Cent	44.6	6.8	27.5	0.1	2.9	10.6	7.5
Lipase neg strains	No 511	180	32	224	0	0	45	30
	Per Cent I	35.3	6.3	43.7			8.8	5.9
	Per Cent II	23.3	27.1	46.8			24.4	23.1
Fibrinolysin neg strains	No 68	29	2	13	0	0	14	10
	Per Cent I	42.7	2.9	19.1			20.6	14.7
	Per Cent II	3.7	1.7	2.7			7.6	7.7

Per Cent I Percentage of total No of negative reacting strains

Per Cent II Percentage of negative reacting strains related to total No of strains isolated within the different phage groups

RESULTS

Table 2 illustrates the enzyme production of the staphylococcal strains. Without exception all these strains produced hyaluronidase and phosphatase, while a few per cent of the strains did not produce fibrinolysin. Lipase could not be demonstrated in more than a quarter of the coagulase positive strains.

One-hundred coagulase negative staphylococcal strains have been used as control material. None of these strains produced hyaluronidase or fibrinolysin. A quarter of the strains produced phosphatase, but the reactions after incubation for 18-22 hours were very weak, giving only a pink colour, compared with the scarlet of the coagulase positive strains. In our protocol we have recorded these strains as \pm , but this is questionable and it is more probable that they should be regarded as negative. About one tenth of the coagulase negative strains gave a positive lipase reaction.

Table 3 shows the distribution of the coagulase positive strains with regard to the origin of the samples. About one-half of the 1,738 strains were isolated from pus and about a quarter of the strains were found in cultures from nose and/or throat swabs. The series shows very little difference in the occurrence of lipase negative and fibrinolysin negative strains in relation to its origin.

Table 4 demonstrates the distribution of the coagulase positive strains according to negative reactions and phage groups. In our earlier examinations (3, 14-16) group III was without doubt the predominant phage group. In our latest examinations, however, there has been a tendency to a steady increase in group I. In the present series group I predominates, comprising more than two-fifths of the coagulase positive strains, while group III is the second largest group accounting more than a quarter of the strains. The low percentage of non-typable strains (7.5 per cent) in this series is also remarkable.

In as many as 511, or not far from one-third of the strains, we have not succeeded in demonstrating lipase production. Not far from a quarter of the strains belonging to group I are lipase negative, and in group III the lipase negative strains approach half of the strains within this group. This difference is significant ($0.01 > \beta > 0.001$).

In the miscellaneous group and among the non-typable strains, the percentages of fibrinolysin negative strains are higher than in phage groups I to III, but the number of strains in the different groups is too small to say if the differences are real.

DISCUSSION

In this series we have, by means of the decapsulation test, found total concordance between the strains producing hyaluronidase and coagulase. Kaffka (6), using the same test, suggests that the demonstration of hyaluronidase is equivalent to the coagulase test, and Oehring (9) using *Bact. amritatum* as test organism, found that the decapsulation test was more frequently positive than the plasma coagulase reaction and plasma agglutination. Faber & Rosendal (2) in a series consisting of 791 strains of coagulase positive staphylococci, found, estimating the hyaluronidase activity by the turbidimetric method, that 97.1 per cent of the strains were able to produce the enzyme.

With regard to phosphatase Barber & Kuper (1) stated that it seems clear that cultures of staphylococci giving a negative phosphatase reaction may be regarded as coagulase negative, and, therefore, presumable non-pathogenic, while only occasional phosphatase positive staphylococcal colonies will be found to be coagulase negative. Kaffka (6) in a series of 277 coagulase positive staphylococcal strains, found that all produced phosphatase, while 9 out of 123 coagulase negative strains showed this property. Without exception all the coagulase positive strains in our material produced phosphatase. With regard to the coagulase negative strains, one fourth gave a weak positive reaction. Here further investigations are needed. It is possible that a more fixed incubation period may be of importance.

Kaffka (6) found that 3.6 per cent of a series consisting of 277 coagulase positive strains did not produce fibrinolysin. This finding agrees well with our results (3.9 per cent). While none of our coagulase nega-

tive strains produced fibrinolysin, Kaffka found two of 121 coagulase negative strains to be fibrinolysin positive

Jessen *et al* (4) suggest that the ability to split Tween usually coincides with a positive egg yolk reaction. In 437 strains investigated with respect to both the egg yolk reaction and the splitting of Tween 80 and Tween 20, these methods gave identical results in 97 per cent of the cases. The same authors (5) have noticed that cases of bacteraemia, caused by egg yolk negative strains, had a distinctly higher mortality rate than cases due to egg yolk positive strains. Parker (10) has stressed that staphylococcal strains which gave a negative egg yolk reaction and a positive serum opacity reaction occurred almost exclusively in superficial lesions. The majority of them were members of type 71, or were penicillin resistant members of phage group III. It is suggested that these organisms can cause superficial inflammation, but are usually unable to invade deeper tissues. Of the strains belonging to phage group II in our series, very few were lysed by phage 71 only. The great majority of lipase negative strains was found in phage group III and most of them were resistant to penicillin.

SUMMARY

A series of 1,738 coagulase positive staphylococcal strains which were isolated from the routine material, sent to the laboratory for bacteriological examination during 1959, has been tested by simple methods to demonstrate the presence of hyaluronidase, phosphatase, fibrinolysin and lipase.

Without exception all these strains produced hyaluronidase and phosphatase, while no fibrinolysin production could be demonstrated in 99 per cent or lipase in 29.4 per cent. The great majority of lipase negative strains was found in phage group III and most of them were resistant to penicillin. One hundred coagulase negative staphylococcal strains were used as control material. None of these strains produced hyaluronidase or fibrinolysin while about one tenth gave a positive lipase reaction. After incubation at 37° C for 18-22 hours a coagulase

may be of importance

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A LONG-TERM STAPHYLOCOCCAL STUDY

By

TH. M. VOGELSAUG and JOHN BØE

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Most investigators in recent years have found a staphylococcal carrier rate in the upper respiratory tract varying between 30 and 70 per cent. As a rule, these investigations are based on the findings in the anterior nares of adult patients in hospital (14). The frequency of carriers among members of hospital staffs may be higher, up to 92 per cent (8).

We have, in our earlier investigations, extended the studies of the carrier rate of pathogenic staphylococci in the upper respiratory tract to include throat carriers. Some of these studies are recorded in Table 1. As will be seen from the table, the carrier rates varied between 60 per cent and 82 per cent. If we had not extended the examinations to include the throat, we would have missed more than one fifth of the staphylococcal strains isolated.

Investigations were carried out on the basis of sporadic findings. In addition we have, during recent years, also made long term studies on

TABLE 1
Carrier Rates of Pathogenic Staphylococci in Earlier Sporadic Studies

Year	Groups	No. of in individuals	Percentage of Staphylococcal Carriers in			Total
			Nose and Throat	Nose only	Throat only	
1951 (16)	Healthy School Children	1000	47	19	17	83
1953 (15)	Hospital Staff	764	40	17	19	76
1954 (7)	Children on Admission to Hospital	1346	46	14	14	74
	Children on Discharge from Hospital	633	54	12	13	79
	Healthy Mothers	71	32	31	14	77
	Hospital Staff	63	37	22	16	75
1959 (19)	Hospital Staff	845	33	17	23	73
1959 (18)	Aged Patients	190	41	23	17	71
	Hospital Staff	106	34	17	18	69
	Total	5018	43	16	17	76
	Percentage of Positive Findings		57	21	22	

TABLE 2

Carrier Rates of Pathogenic Staphylococci in Earlier Long Term Studies

Year	Groups	No of Individuals	No of Examinations	Percentage of Positive Swabs in		Types of Staphylococcal Carriers		Non Carriers
				Nose	Throat	Persistent	Intermittent	
1956 (11)	Children	67	505	49	59	10	45	12
1958 (17)	Medical Students	42	492	27	45	16	21	5
1960 (3)	Hospital Staff	100	1146	35	48	34	54	12
	Total	209	2044			60	120	29
	Percentage					29	57	14

the carrier state in the University Hospital, Bergen. Findings from these studies are recorded in Table 2.

Sixty-seven children were swabbed at intervals of one or two days (11). Only six children were less than one year of age, 27 between one to six years old, while about 50 per cent of the children were of school age. Of the ten permanent carriers, none were found in the first group, 4 were found in the age group one to six years, 6 were school-children

Another study was made on the carrier rate of pathogenic staphylococci in two groups of medical students during their training in the medical and surgical departments (17). Only two students were permanent carriers both in the nose and in the throat. If we take these two sites separately, five students were found to be permanent carriers of staphylococci in the nose, 11 in the throat.

A long-term study has recently been made on the carrier rate of pathogenic staphylococci in the nose and in the throat in members of the staff in the same department in which the present investigations

TABLE 3

Number of Swabs of Each Individual

Individuals																				Total	
No of Examinations		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18		
<hr/>																					
Members of the Staff																					
No of Individuals		27	5	4	2	2	3	2	2	3	3	3	1	3	6	3	3	3	3	75	
Total No of Examinations		54	15	16	10	12	11	16	18	30	33	36	11	42	90	48	51	54	559		
<hr/>																					
Patients																					
No of Individuals		75	50	13	5	2															147
Total No of Examinations		75	100	39	20	10															246

were carried out (3) Five to 15 swabs were taken from 100 individuals, 34 were permanent, 54 intermittent staphylococcal carriers. Ten permanent and 12 intermittent carriers would have been missed if the throat had not also been swabbed. Of the 980 pathogenic strains isolated, only 10 per cent were resistant to penicillin and 9 per cent to streptomycin. Only 48 per cent of the isolated strains were typable by the Routine Test Dilution (RTD), whereas 39 per cent were lysed by employing phages at 1,000 times the concentration used in the RTD. Phage group III was the predominant group amounting to 36 per cent followed by group I amounting to 26 per cent. The permanent carriers had a tendency to retain strains of the same antibiograms and phage patterns which impeded the implantation of other strains.

The present study is a continuation of these investigations.

MATERIAL AND METHODS

The material consists of 75 members of the staff and 147 patients in the Medical Department B, University Hospital, Bergen.

The methods used for the isolation of the staphylococcal strains, antibiogram determination and phage typing, have been described in earlier communications (3-5).

RESULTS

1. Carrier Rate

Table 3 shows the number of swabs removed from the individual members of the staff. Ten or more swabs from nose and throat were taken weekly in 28 cases, 5 to 9 swabs in 11, and less in 36 cases. Most of these last mentioned individuals were trainee nurses, who were on duty in the department for a shorter time, and were either transferred to other departments during the examination period, or started their work in the department within the same period.

The patients were swabbed for considerably shorter periods, most of them only 1-2 times with an interval of a week. This is due to the fact that in a modern medical department there is a rapid turn over of patients and hence stays in hospital are shorter now.

Table 4 illustrates the occurrence of pathogenic staphylococci in the material. Out of the total number of 559 swabs obtained from members of the staff, pathogenic staphylococci were isolated in 344 cases, i.e. 62

TABLE 4
Occurrence of Pathogenic Staphylococci

Individuals	No of In-lab swabs taken	Total no. of swabs taken	Positive Swabs					Negative swabs	Per cent
			Nose and Throat	Nose only	Throat only	Total	Per cent		
Members of the Staff	75	519	100	69	175	344	62	215	38
Patients	147	246	63	32	34	129	50	117	48

1960

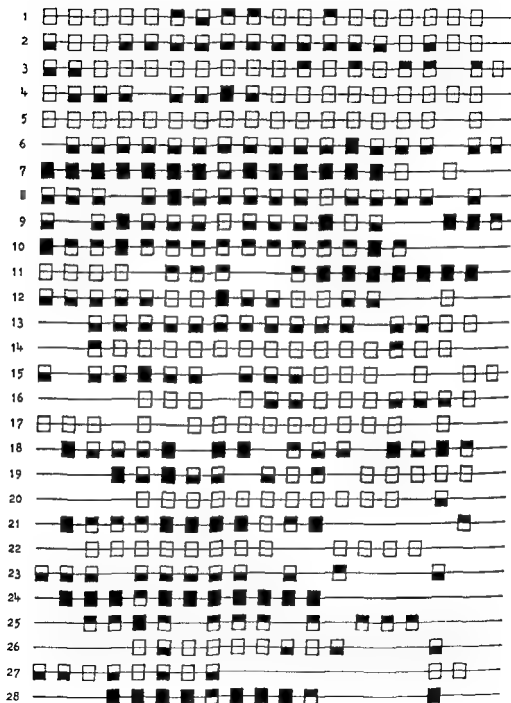
 $10/5$ $17/5$ $24/5$ $31/5$ $7/6$ $21/6$ $28/6$ $5/7$ $12/7$ $19/7$ $25/7$ $2/8$ $9/8$ $16/8$ $27/8$ $30/8$ JULY AUG SEPT


Fig 1

The carrier histories of the 28 members of the staff swabbed 10-18 times

per cent while out of 246 examinations of patients 129, i.e. 52 per cent, gave positive results. This difference between staphylococcal findings in staff and patients is ascribable mainly to the higher number of members of the staff harbouring pathogenic staphylococci in the throat.

On two occasions members of the staff were found to harbour two staphylococcal strains in the nose, on eleven occasions in the throat. As a result of these duplicates the material obtained from members of the staff consists of 171 strains isolated from the nose, and 286 strains from the throat, adding up to a total of 457 staphylococcal strains.

Correspondingly, two patients harboured 2 staphylococcal strains in the nose, and three in the throat. Hence, the material isolated from the patients consists of 97 strains from the nose, and 100 strains from the throat, 197 staphylococcal strains in all.

Fig. 1 shows the carrier histories of the 28 members of the staff who were swabbed 10-18 times. In 3 of these cases no pathogenic staphylococci were demonstrable during the entire examination period. Seven members presented growth of pathogenic staphylococci from the nose and/or the throat on all examinations. On one occasion three members presented no pathogenic staphylococci neither in the nose nor in the throat although both preceding and subsequent swabs showed growth of pathogenic staphylococci of the same phage patterns and with the

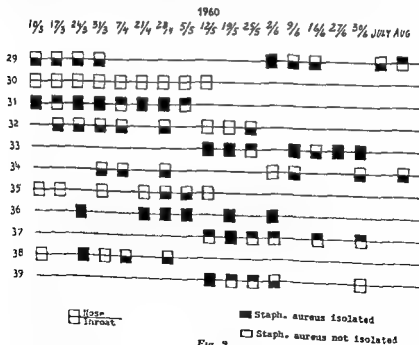


Fig. 2

The carrier histories of the 11 members of the staff swabbed 5-9 times

same antibiograms. In two members pathogenic staphylococci were not demonstrable at the two last examinations in this period. Both have later been re-examined, and presented growth of pathogenic staphylococci of the same phage patterns and antibiograms as in all the earlier positive examinations. For these reasons the negative findings have been regarded as technical failures, and the two cases have been included in the group of permanent carriers. Besides it will be noticed that these two members were included in the preceding study (3, 5) when one was examined 9 and the other 12 times, on each examination both harboured staphylococcal strains of the same phage patterns and antibiograms as discussed in the present study.

In periods fourteen of the members would be carriers of pathogenic staphylococci, alternating with periods of non-carriage. In one member staphylococci were isolated only on one occasion and in another member on two occasions. Therefore, of the 28 members listed in Fig. 1, 12 are considered to be permanent and 11 intermittent carriers, while 5 are regarded as trivial carriers or non-carriers.

Fig. 2 shows the carrier histories of the 11 members of the staff who were swabbed 5-9 times. Five of these members are regarded as permanent carriers and a similar number of members as intermittent carriers, while no pathogenic staphylococci were demonstrable in one member.

Of the members swabbed 5 times or more, 17 were permanent and 16 intermittent carriers, while 6 were trivial carriers or non-carriers.

Only in one carrier staphylococci were present constantly both in the nose and the throat during the whole examination period. While 6 members were constant nose carriers, only one carrier constantly harboured staphylococci in the throat. In the intermittent carriers 45 per cent of the swabs from the throat gave positive findings, compared to only 15 per cent of the swabs from the nose.

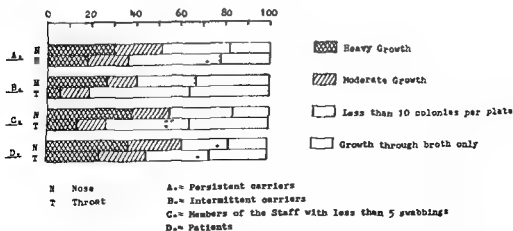


Fig. 3

Growth rates of the pathogenic staphylococci isolated from the carriers

In Fig. 3 the material is divided into different groups and shows the degree of growth from the nose and the throat separately. In all four groups growth of pathogenic staphylococci from the nose is by far more marked than growth from the throat. Both in members of the staff who were swabbed less than 5 times and also in the material of patients more than one third of the positive findings in the nose showed heavy growth of pathogenic staphylococci. One fourth of the staphylococcal strains isolated from the throats of patients showed heavy growth while only 11 per cent of the corresponding strains isolated from the throats of the intermittent carriers gave these findings.

2 Antibigram Determinations

The disc method has been employed to determine the resistance to sulphathiazol and the following 5 antibiotics: Penicillin, streptomycin, tetracycline, erythromycin and chloramphenicol. +++ or ++ was regarded as sensitive and + or 0 as resistant.

None of the staphylococcal strains were resistant to chloramphenicol and only one single strain isolated from the nose of a member of the staff was resistant to erythromycin.

Table 5 illustrates the resistance determinations to sulphathiazol and to the 3 other antibiotics mentioned.

Sulphathiazol Less than one fifth of the strains isolated from members of the staff were resistant to this drug while only 13 per cent of the strains isolated from patients showed resistance.

Penicillin Almost three fourths of the staphylococcal strains isolated from the members of the staff were resistant to this antibiotic. There were more resistant strains among the permanent than among the intermittent carriers. Less than 50 per cent of the strains isolated from the patients were resistant to this antibiotic.

Streptomycin About one tenth of the staphylococcal strains showed resistance to this antibiotic. The number was somewhat higher among strains isolated from the patients than among the strains isolated from the members of the staff.

Tetracycline Most of the staphylococcal strains were sensitive to this antibiotic.

Of the 457 staphylococcal strains isolated from members of the staff 343 or 75 per cent showed resistance. Of these strains 262 were resistant only to one of the chemotherapeutics: 50 to 2, 23 to 3 and only 8 to 4 (Table 6).

Less than 50 per cent of the 197 staphylococcal strains isolated from the patients were resistant: 74 to 1 of the chemotherapeutic agents, only 1 single strain to 2, 9 to 3 and 10 to 4.

Table 7 records the different combinations of resistance. Of the strains isolated from members of the staff 3 were resistant to 4 drugs. Seven of these strains were resistant to sulphathiazol, penicillin, strepto-

TABLE 5
Resistance Determination to Sulphathiazol Penicillin Streptomycin and Tetracycline

Groups	Sites	No of Strains	Percentage													
			Sulphathiazol			Penicillin			Streptomycin			Tetracycline				
			+++ or ++	+	0	+++ or ++	+	0	+++ or ++	+	0	+++ or ++	+	0		
Permanent Carriers	Nose	116	67	20	13	33	11	67	22	89	84	3	13	16	98	2
	Throat	134	80	16	4	20	33	50	17	67	90	2	8	10	98	2
	Total	270	74	18	8	26	24	57	19	76	87	3	10	13	98	2
Intermittent Carriers	Nose	31	94	3	3	6	42	45	13	58	97		3	3	90	10
	Throat	91	90	9	1	10	30	53	17	70	100				100	
	Total	122	91	7	2	9	33	51	16	67	99		1	1	97	3
< 5 Swabs	Nose	24	88	4	8	12	29	21	50	71	92	4	4	8	100	
	Throat	41	98		2	2	34	44	22	66	100				100	
	Total	65	94	1	5	6	32	36	32	68	97	1	2	8	100	
Members	Nose	171	75	15	10	25	19	56	25	81	88	12	10	12	97	2
	Throat	286	86	11	3	14	32	50	18	68	94	1	6	6	99	1
	Total	457	82	12	6	18	27	53	20	73	92	2	6	8	98	2
Patients	Nose	97	88	7	5	12	59	27	14	41	90	2	8	10	95	5
	Throat	100	86	8	6	14	55	28	17	45	88	2	10	12	95	5
	Total	197	87	7	6	13	57	27	16	43	89	2	9	11	95	5

TABLE 6

Number of Staphylococcal Strains Resistant to one or more Antibiotics

	Resistance to no. of Antibiotics				No. of Strains	Percentage of Resistance
	1	2	3	4		
Groups						
Members of Staff	262	50	23	8	343	75
Patients	74	1	9	10	94	48

TABLE 7
Combinations of Resistance

S	P	Str	T	E	No. of Strains from	
					Members	Patients
X	X	X	X		7	10
	X	X	X	X	1	
X	X	X			23	9
X	X				50	9
X					4	8
	X				251	65
		X			7	3
					343	94

S Sulphathiazol P Penicillin Str Streptomycin
T Tetracycline E Erythromycin

mycin and tetracycline Twenty three strains were resistant to the 3
ioned and 50 resistant only to sul-
strains which were resistant to one
ty, viz 252, were resistant to peni-
cillin only

Of the strains isolated from the patients, the corresponding numbers
were 10, 9, and 1 Of the 94 strains resistant only to one chemothera-
peutic agent, 65 were resistant to penicillin only

3 Phage Typing

A certain change occurred in phages used in the present study
as compared with the earlier examinations (5, 20) Two new phages
have been added, 81 and 82, and phage 73 has been excluded Hence the
series include 22 phages, which are divided into groups listed in
Table 8

The differences from the earlier basic set of phages are found in
group I which now consists of 7 as against 5 phages, and in group III
where the phages are reduced from 11 to 8 Some of the phages may
overlap from one group to another, especially group I and III, and
the strains which show this overlapping are placed in the "miscellane-
ous" group

TABLE 8
Phages Belonging to the Different Phage Groups

Phage group	Phages in the group
I	29, 52, 52 A, 79, 80, 81, 82
II	3A, 3B 3C 55, 71
III	6, 7, 42E, 47, 53, 54, 75, 77
IV	42D
V	187

TABLE 9
Phage Grouping of the Pathogenic Staphylococci

Phage groups	Member of the Staff		Patients	
	No	Per cent	No	Per cent
I	101	22	54	27
II	61	14	9	5
III	183	40	87	44
V	27	6	13	7
Misc	34	7	16	8
NT	51	11	18	9
Total	457	100	197	100

Misc - Miscellaneous group NT - Non-typable

Table 9 shows the distribution of the pathogenic staphylococcal strains within the different groups. There is rather good correlation between results obtained among strains isolated from the members of the staff and strains isolated from patients. In both groups about one-tenth of the strains were non-typable both by the RTD and the concentrated phages. The other strains were typable on the basis of the lytic reactions of the 22 phages contained in the present set. Group III was the predominant group in both series including about two-fifths of the strains, while about one-fourth of the strains belonged to group I. Differences between strains isolated from the members of the staff and from patients are most marked in group II, where 14 per cent of the strains isolated from members and 5 per cent only of the strains isolated from patients belonged to this group. Group IV (42D) was not found in this series.

4 Relationship Between Phage Typing and Penicillin Resistance

Figs. 4 and 5 illustrate the distribution of the staphylococcal strains according to penicillin resistance and phage grouping. There is, on the whole, good agreement between the 2 figures. Staphylococcal strains resistant to penicillin were found in all of the phage groups recorded, most frequently in group III and in group I. The only exception is group V (type 187). None of the 13 staphylococcal strains belonging to this group and isolated from patients were resistant to penicillin.

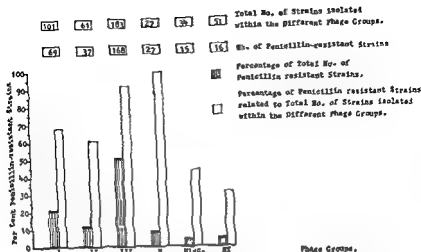


Fig 4

Distribution of the staphylococcal strains isolated from the members of the staff according to penicillin resistance and phage grouping

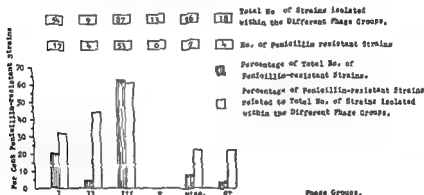


Fig 5

Distribution of the staphylococcal strains isolated from the patients according to penicillin resistance and phage grouping

Conversely, 27 staphylococcal strains isolated from members of the staff and belonging to this group, were all resistant to penicillin. Both in group I and III, the percentage of penicillin resistant strains as related to the total number of strains isolated within this group, was higher among the strains isolated from the members of the staff than among the strains isolated from the patients.

Table 10 illustrates the antibiogram determination of the strains belonging to the different phage patterns within the phage types 52 72 180 81 82 (group I).

Of the 81 strains within these phage patterns, 23 were sensitive to all drugs, 16 were resistant to 1, 15 to 2, 22 to 3 and 5 to 4 antibiotics.

TABLE 10

Antibiogram Determination of the Strains Isolated by 80/81/82 and Related Phages

Phage Patterns	No of Strains	Antibiogram Determinations						No of Strains
		S	P	Str	T	E	Chl	
82	1	X	0	X	X	X	X	1
81/82	11	X	X	X	X	X	X	5
		X	0	X	X	X	X	6
80/81/82	31	X	X	0	X	X	X	1
		0	0	X	X	X	X	9
		0	0	0	X	X	X	16
		0	0	0	0	X	X	11
52/81	1	X	X	X	X	X	X	1
52/81/82	2	X	X	X	X	X	X	2
52/52A/80	10	X	X	X	X	X	X	2
		0	0	X	X	X	X	6
		0	0	0	X	X	X	2
52/52A/81	1	X	X	X	X	X	X	1
52/52A/81/82	6	X	X	X	X	X	X	2
		X	0	X	X	X	X	4
52/52A/80/81/82	18	X	X	X	X	X	X	10
		X	0	X	X	X	X	4
		0	0	0	X	X	X	4
	81							81

S - Sulphathiazol P - Penicillin Str - Streptomycin
 T - Tetracycline E - Erythromycin Chl - Chloramphenicol
 0 - Resistant X - Sensitive

Most of the strains with combined resistance belonged to the 2 patterns 80/81/82 and 52/52A/80 81/82

DISCUSSION

These examinations were carried out during a period in which working conditions of the staff were very difficult, because of the comprehensive rebuilding and modernisation of the department taking place at this time. The large wards were changed into small units. Indeed, under such circumstances it has been very hard to maintain any high hygienic standard. On the other hand, spread of infections within the wards has, in the main, been avoided. Although some of the patients have been infected with pathogenic staphylococci during their stay in hospital, no specific hazard of staphylococcal infection has existed.

Pathogenic staphylococci were isolated from 62 per cent of the members of the staff and 52 per cent of the patients. These carrier rates are in good conformity with the findings in the earlier long term studies (3). Staphylococci were found more often in the throat than in the nose, but growth from the throat has mostly been scanty. From the nose there has been a markedly heavier growth of pathogenic staphylococci both in members of the staff and in patients. There is, therefore, reason to believe that the nasal carriers play a more promi-

nent part than the throat carriers as regards the spread of staphylococcal infections

Of the 75 members of the staff, 39 were swabbed from 5 to 18 times. Seventeen of these members are regarded as permanent carriers who retained the same strains during the whole examination period. The permanent carriers have, therefore, a tendency to resist implantation of other strains. If the permanent carriers do not harbour the staphylococcal strain in question, this strain will not have the same chances of colonizing the throat and the nose as it would have in intermittent carriers.

TABLE 11
Resistance to Penicillin and Streptomycin of the Staphylococcal Strains Isolated in the Medical Department at Different Times

In Individuals	Series	No. of Strains	Percentage of Resistance to	
			Penicillin	Streptomycin
Members of the Staff	1957-1958	158	36	24
	1960	969	36	10
Patients	Present	197	43	11
		457	73	8

Antibiogram determination of the staphylococcal strains isolated from members of the staff in the medical department was performed in 1957-1958 (20) and in 1960 (4) using exactly the same technique as in the present study. The results obtained should therefore be directly comparable and are illustrated in Table 11.

The examination of the members of the staff carried out in 1957-1958 gave a high incidence of penicillin resistant staphylococci and about one fourth of the strains were resistant to streptomycin. As a result of these findings the use of antibiotics in the department has become more reserved which is reflected in the lower incidence of resistant strains seen in 1960. In the present study, the incidence of streptomycin resistant strains is low. However, about three-fourths of the strains isolated from members of the staff were resistant to penicillin, while only about two fifths of the strains isolated from patients were resistant to this antibiotic. The great reservation with which penicillin is used and the individual calculation of the dosages have been continued. The present increasingly higher incidence of resistant strains seen in members of the staff, therefore, must have other causes. Some of the resistant strains may have been transferred to the medical department from other departments where the incidence is high.

... have represented a contributory cause in

TABLE 10

Antibiogram Determination of the Strains Isolated by 80/81/82 and Related Phages

Phage Patterns	No of Strains	Antibiogram Determinations						No of Strains
		S	P	Str	T	I	Chl	
82	1	X	II	X	X	X	X	1
81/82	11	X	X	X	X	X	X	5
		X	0	X	X	X	X	II
80 81/82	31	X	X	0	X	X	X	1
		0	II	X	X	X	X	9
		0	0	0	X	X	X	16
		0	0	0	0	X	X	5
52/81	1	X	X	X	X	X	X	1
52/81/82	2	X	X	X	X	X	X	2
52/52A/80	10	X	X	X	X	X	X	2
		0	0	X	X	X	X	6
		0	II	0	X	X	X	2
52/52A/81	1	X	X	X	X	X	X	1
52/52 1/81/82	6	X	X	X	X	X	X	2
		X	0	X	X	X	X	4
52/52 1/80/81/82	18	X	X	X	X	X	X	10
		X	0	X	X	X	X	4
		0	II	0	X	X	X	4
	81							81

S - Sulphathiazol P - Penicillin Str - Streptomycin
 T - Tetracycline F - Erythromycin Chl - Chloramphenicol
 0 - Resistant X - Sensitive

Most of the strains with combined resistance belonged to the 2 patterns 80 81/82 and 52/52A/80 81/82

DISCUSSION

These examinations were carried out during a period in which working conditions of the staff were very difficult, because of the comprehensive rebuilding and modernisation of the department taking place at this time. The large wards were changed into small units. Indeed, under such circumstances it has been very hard to maintain any high hygienic standard. On the other hand, spread of infections within the wards has, in the main, been avoided. Although some of the patients have been infected with pathogenic staphylococci during their stay in hospital, no specific hazard of staphylococcal infection has existed.

Pathogenic staphylococci were isolated from 62 per cent of the members of the staff and 52 per cent of the patients. These carrier rates are in good conformity with the findings in the earlier long-term studies (3). Staphylococci were found more often in the throat than in the nose, but growth from the throat has mostly been scanty. From the nose, there has been a markedly heavier growth of pathogenic staphylococci both in members of the staff and in patients. There is, therefore, reason to believe that the nasal carriers play a more promi-

They were isolated repeatedly from 2 permanent and 2 intermittent carriers. Conversely, only 13 strains belonging to this type were isolated from patients and none of these were found resistant to penicillin.

SUMMARY

A long term study has been made of the carrier rates of pathogenic staphylococci in the upper respiratory tract of 75 members of the staff and 147 patients in the Medical Department B University Hospital, Bergen.

Repeated swabs were taken from the nasal cavities and from the throat at weekly intervals. Out of 559 swabs obtained from members of the staff, pathogenic staphylococci were isolated in 62 per cent, while out of 246 examinations of patients, 52 per cent gave positive results.

Of the members of the staff swabbed 5 times or more, 17 were permanent and 16 intermittent carriers, while 11 were trivial carriers or non carriers. While 6 members were constant nose carriers, only one carrier constantly harboured staphylococci in the throat. Of the intermittent carriers, 45 per cent of swabs taken from the throat gave positive findings, compared to only 15 per cent of the swabs from the nose. Swabs from the nose, however, showed a more marked growth of pathogenic staphylococci whether obtained from the members of the staff or from the patients.

About three fourths of the strains isolated from members of the staff were resistant to penicillin while only about two fifths of the strains isolated from patients were resistant to this antibiotic. The lower incidence seen among the patients may be explained by their short stays in hospital.

Next to penicillin, tetracycline has been the antimicrobial drug of choice in the department. Although most of the staphylococcal strains were sensitive to this antibiotic, the incidence of resistant strains was somewhat higher among the strains isolated from patients than among those isolated from members of the staff.

None of the staphylococcal strains were resistant to chloramphenicol and only one strain, isolated from the nose of a member of the staff, was resistant to erythromycin.

Phage typing showed rather satisfactory correlation between results obtained among strains isolated from members of the staff and from patients. In both groups about one tenth of the strains were non-typable. Group III was the predominant group in both series including about two fifths of the strains, while about one fourth of the strains belonged to group I. More than 50 per cent of the strains belonging to group I were lysed by phages 80/81 or related phage patterns. Staphylococcal strains resistant to penicillin were found in all the phage groups, most frequently in group III and in group I.

the present increase of incidence. The lower incidence seen among patients may be explained by their short stay in hospital. Another study of 297 patients admitted to the same department showed a steady rise in incidence of carriage of antibiotic resistant staphylococcal strains during their stay in hospital (6).

In the period in which these examinations were carried out tetracycline has been, next to penicillin, the antimicrobial drug of choice in the department. Although most of the staphylococcal strains have been found sensitive to this antibiotic, the incidence of resistant strains has been somewhat higher among the strains isolated from patients than among strains isolated from members of the staff.

Only one staphylococcal strain has been found resistant to erythromycin and all the isolated strains have been found sensitive to chloramphenicol. Use of the former in the department has been very limited and reserved for vital indications. Chloramphenicol has been used more freely, especially for the treatment of croup patients in the isolation ward.

Group III was the predominant phage group both among the staphylococcal strains isolated from the members of the staff and from the patients, consisting of 53 per cent and 44 per cent of the strains in the whole series respectively. Of the 332 penicillin resistant strains isolated from the members of the staff, 50 per cent belonged to group III, while the corresponding percentage of penicillin resistant strains isolated from patients was 62.

This preponderance of penicillin resistant group III strains is in good conformity with the findings in earlier investigations in the same department (4,20). Group III has also been the predominant group in other widely separated geographical areas (5).

The now well-known strain of group I, type 80, appeared in Australia in 1954 (13). Type 80 and the related type 81 (2) has later gradually replaced group III strains as the causal organism of hospital infections, and group I strains have in recent years been found to be the predominant phage group in Germany (9, 10). It has been demonstrated (1, 12) that artificial lysogenization of the typical 80/81 strain with certain phages can convert the typing pattern to 52/52A/80/81, a related pattern often isolated with typical 80/81 strains in the same outbreak. Of the 155 staphylococcal strains belonging to group I in the present series, a total of 81 were lysed by 80/81 and related phage patterns. In a long-term examination, the percentages of strains lysed by the different phages, depend upon the ratio of permanent to intermittent carriers. If some permanent carriers are harbouring strains lysed by the phages within a special group, these strains will be isolated repeatedly and the percentage of such strains will, therefore, be higher than in a single investigation.

Of the staphylococcal strains isolated from the members of the staff 27 were lysed by type 187 and they were all resistant to penicillin.

ELECTRON MICROSCOPIC OBSERVATIONS ON THE MUCUS PRODUCTION IN HUMAN AND RAT INTESTINAL GOBLET CELLS

By

I. BIERRING

Received 30 vi 61

Electron microscopy of gland cells permits observation of formative stages in the elaboration of secretory products in the form of secretory granules. Topographical studies of the relationships between the granulogenic stages and the cell organelles have suggested the role played by these cell elements in secretory activity. The comprehensive morphological evidence about this is partly derived from the efforts to establish the ultrastructural identity of the cell organelles (Sjostrand & Hanzon 1954, Dalton & Felix, 1956, review by Haguenau 1958).

To some extent, or rather with regard to certain types of gland cells, the information gained from electron microscopic studies lends support to the classic conception (Lassanow 1923, 1924, Bowen 1926, 1929) of the participation of the Golgi apparatus in the formation of the secretory products. This is true of, amongst others, the mucus producing gland cells.

The formation of mucoid granules within the Golgi apparatus was observed in the goblet cells of the tracheal epithelium by Rhodin (1959) and in the mucous cells of the salivary glands by Scott & Pease (1959) and Scott (1959). Palay (1958), in his review of "The morphology of secretion", gives a precise description of the same phenomenon occurring in goblet cells from the jejunal epithelium of a rat. Later, however, Taylor (1959) reported a different pattern in the development of mucoid granules in intestinal goblet cells of a rat; he describes the granules as pinched-off, circumscribed dilations of the ergastoplasm bounded by ergastoplasmic membranes. The present study, therefore, is an attempt to re-evaluate the role of the cell organelles in the production of mucus in the intestinal goblet cells. In view of possible species differences in the mechanism of cellular secretion, both human and rat intestinal mucosa were examined.

Radioautographic studies following the injection of tritium labelled thymidine (Leblond & Messier 1958) have confirmed that the intestinal goblet cells are formed in the deep parts of the crypts by prolifera-

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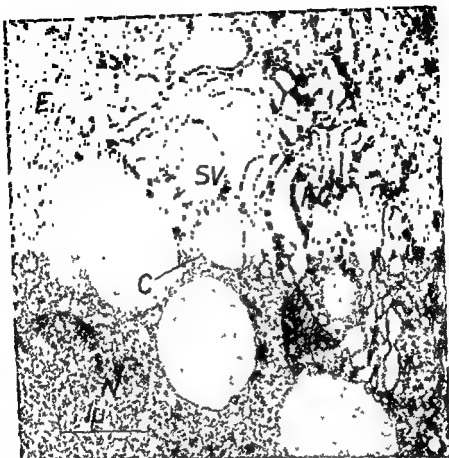


Fig 2

A higher power electron micrograph of the supranuclear area of the goblet cell, which is seen to the left in Fig 1. Small almost circular vesicles (SV) are scattered within the area. FC, flat cisterna; C, dilated cisterna. The smooth character of the membranes limiting the cisternae, is emphasized. The change in the density of the interior of the cisternae which accompanies increasing external calibre is illustrated. N, nucleus. Two secretory granules are found above the nucleus, between them a dilated round cisterna is seen. E, ergastoplasm.

tion of goblet cells. With increasing maturation they migrate in the direction of the summit of the villus, to be finally shed there. Goblet cells whose cytoplasm, as a result either of scanty production or of excessive excretion contain only few secretory granules, offer optimal possibilities for the study of the participation of the cell organelles in the secretory process. Nearly empty goblet cells are most easily found in the form of young goblet cells, presenting initial stages of secretion, and the present study is concentrated accordingly on goblet cells from regions near the base of the crypt.

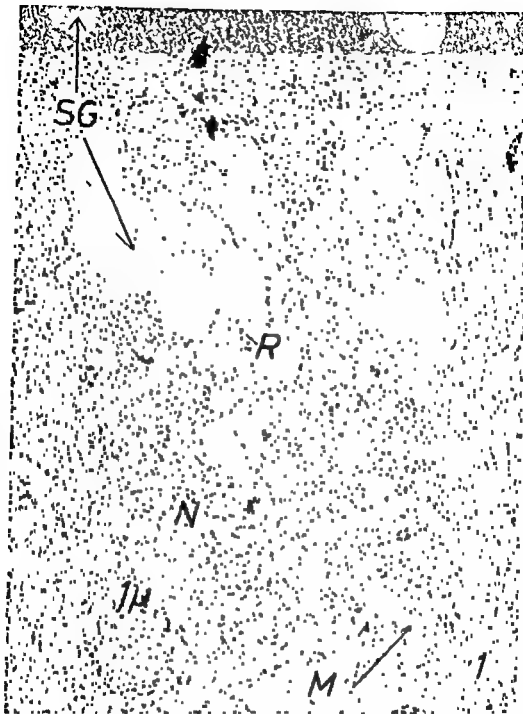


Fig 1

The basal part of two young twin goblet cells from a jejunal crypt (rat). SG mature secretory granules. The vesicular complex occupying the supranuclear area is dominated by large vesicles or cisternae. The form of the cisternae range from flattened to almost circular. Many of the cisternae may be interpreted as representing transitional stages between flattened cisternae and mature secretory granules. N nucleus, M, mitochondria, H accumulations of RNA granules.

other, often at one or at both ends of the cisterna. Thus, localized dilations are seen. Adjoining these, more diffusely dilated cisternae can be seen, oval or nearly circular in shape, more and more assuming the form of the mature secretory granule. In one of the cells in Fig 1, this change in form can be observed from the peripheral part of the supranuclear field towards its centre, in the other cell, from one side of the field towards the other. Judging from their form alone, most cisternae thus represent transitional stages between the flat and the round types. This impression receives further support from the changes which can be observed within the cisternae with increasing calibre. The interior of the flat, or nearly flat, cisternae is clear. With increasing calibre the density of the interior also increases, first in the form of a dispersed, floccular pattern on a background of low density, and gradually showing a diffuse increase in density. The interior of the large cisternae resembles exactly that of the secretory granules. Just above the basally sited nucleus in Fig 2, two mucoid secretory granules are seen. Between them is a large round cisterna. Morphologically, this differs from a secretory granule by its surface membrane alone. To summarize, it may be said that, morphologically, the secretory granules appear to emerge in the supranuclear region from flattened cisternae, which, under dilatation, increase their content of electron dense material. The final stage in the development is the disappearance of the surface membrane. Vesicular elements resembling stages in the development of secretory granules have not been observed elsewhere in the cell.

Another cytoplasmic structure is seen especially in the peripheral parts of the cell. From its characteristic appearance, it can be identified as the ergastoplasm. It is found opposite the supranuclear zone, stretching down along the sides of the nucleus, between the nucleus and the supranuclear cisternae, and up into the luminal part of the cell. In the luminal part it is displaced towards the plasma membrane by the secretory granules, which in the maturing cell ascend and accumulate here. The ergastoplasm consists of elongated, stacked pairs of lamellae. Each lamellar pair (Fig 3) encloses a tubular space of slightly irregular calibre with a homogenous content of lower density than the cytoplasm between the lamellar pairs. The inner lamellar surfaces forming the walls of these spaces are smooth. The outer lamellar surfaces are rough, owing to the presence of small electron opaque granules adhering to them. Granules of a similar appearance, but not associated with lamellae also occur frequently in the cytoplasm. They usually appear in small aggregations, sometimes in such large numbers within young goblet cells that their cytoplasm is darker than that of the absorptive cells. Granules are also found in the supranuclear region of the cytoplasm.

Ergastoplasmic tubular spaces and dilated cisternae often show a very close relationship. This is illustrated in Fig 4. The tubular space ends, apparently, on the surface of the cisterna. A direct continuity is

METHODS

Human jejunal mucosa was obtained from a patient undergoing gastric resection for peptic gastric ulcer. Immediately following the opening of the abdomen a biopsy specimen of the jejunum was taken at the site of the future gastro enteric anastomosis, i.e. from the proximal part of the jejunum. Thin sections of tissue were quickly cut minced according to Pease's method (1960) and immersed in ice cold 1 per cent OsO_4 solution in acetate veronal buffer (pH about 7.2) (Palade 1952).

The fixative was renewed several times during the first part of the fixation period which lasted $1\frac{1}{2}$ hours. A brief washing in Thyrode's solution was followed by dehydration in ethyl alcohol. The specimens were then transferred to a mixture of *n* butyl methylmethacrylate (9:1) to which 1 per cent benzoyl peroxide has been added as a catalyst. Polymerization temperature was 60°C .

Young albino rats were used in the study of rat intestinal mucosa. Laparotomy was performed under ether anaesthesia. Approximately 2 cm of the proximal part of the jejunum was opened along the antimesenteric border. After carefully drying off the visceral contents and mucus the mucosa was fixed by continuous dripping with ice cold OsO_4 solution for about 20 minutes. After cutting out the specimen of tissue it was further fixed for $1\text{--}1\frac{1}{2}$ hours. The remaining procedure was as described above.

Sections were cut on a LKB microtome and studied in a Philips EM 100 B electron microscope some after further staining with potassium permanganate.

OBSERVATIONS

The most conspicuous feature in the cytoplasm of young secreting goblet cells (Fig 1) is the elongated vesicular complex, comparable in size with the nucleus, occupying the supranuclear region in the cell. In the ultrathin sections this complex is composed of vesicles of various sizes and forms embedded in the cytoplasmic matrix. With higher magnification two types of vesicles can be distinguished (Fig 2). The first type consists of small uniform vesicles, nearly circular, with diameters varying between about 350 and 650 Å, the enclosing membrane appearing as a smooth, dense line, whereas the vesicular content is often of a lower density than the surrounding cytoplasm. This type of vesicle appears in large numbers within the entire supranuclear field, where this is not occupied by the second type, the large vesicles, and is also seen in the cytoplasm adjacent to the outside of the large vesicles. These large vesicles are closely packed in groups, the small type of vesicle is found between such groups of large vesicles, but never between individual members of a group.

The large vesicles, in the following termed cisternae, vary considerably in size and especially in shape, from elongated and very flattened to nearly circular. The cisternae have a limiting membrane with strong electron-scattering power: it is seen as a dense, smooth line without attached granular elements. The very flattened cisternae thus are bounded by two parallel, closely adjacent membranes joining in a curve at either end of the cisterna. The width of a flattened cisterna is greater than the distance between adjacent cisternae. This latter distance is, moreover, constant and independent of the shape of the cisternae.

Cisternae adjacent to flattened cisternae show more irregular form. In places the parallel parts of the limiting membrane diverge from each

other, often at one or at both ends of the cisterna. Thus, localized dilations are seen. Adjoining these, more diffusely dilated cisternae can be seen, oval or nearly circular in shape, more and more assuming the form of the mature secretory granule. In one of the cells in Fig 1, this change in form can be observed from the peripheral part of the supranuclear field towards its centre, in the other cell, from one side of the field towards the other. Judging from their form alone, most cisternae thus represent transitional stages between the flat and the round types. This impression receives further support from the changes which can be observed within the cisternae with increasing calibre. The interior of the flat, or nearly flat cisternae is clear. With increasing calibre the density of the interior also increases, first in the form of a dispersed, floccular pattern on a background of low density, and gradually showing a diffuse increase in density. The interior of the large cisternae resembles exactly that of the secretory granules. Just above the basally sited nucleus in Fig 2, two mucoid secretory granules are seen. Between them is a large round cisterna. Morphologically, this differs from a secretory granule by its surface membrane alone. To summarize, it may be said that, morphologically, the secretory granules appear to emerge in the supranuclear region from flattened cisternae, which, under dilatation, increase their content of electron dense material. The final stage in the development is the disappearance of the surface membrane. Vesicular elements resembling stages in the development of secretory granules have not been observed elsewhere in the cell.

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Fig. 3

Part of the supranuclear area of a non-foliated human jejunal goblet cell. The upper pole of the nucleus is seen at the lower margin of the picture. The rough plasma (rER) is found above the nucleus. The smooth cisternae are displaced toward the periphery of the cell by the mature secretory granules. At least a tubular space is apparently ending on the surface of a cisterna, isolated from the interior of the cisterna by the smooth membrane. At least a tubular space (c) is on the plasma membrane. The interdigitating folds of the plasma membrane are almost straightened out.

not seen, the tubular space is separated from the interior of the cisterna by the cisternal membrane. Tubular spaces ending on the plasma membrane and on the outer nuclear membrane are also frequently seen.

The mitochondria are predominantly sited basally to the nucleus and luminally to the supranuclear zone. Mitochondria are found occasionally between the nucleus and the supranuclear cisternae. Fewer mitochondria are found than in the absorptive cells; they are short, round or oval.

The remaining features in the ultrastructure of the goblet cell need



Fig 5

1 0 μ m full distended human jejunal goblet cell T terminal bars



Fig 5

The luminal part of a human jejunal goblet cell. The luminal surface is partly covered by microvilli, partly smooth, especially over the central part of the surface where the secretory granules are protruding. PM: plasma membrane.

only be summarized. Empty, or nearly empty, goblet cells are columnar or prismatic, like the absorptive epithelial cells. The plasma membrane of the sides facing the other epithelial cells exhibits extensions of the interdigitating or interlocking type, their course is less complicated than that of the interdigitating folds seen between the absorptive cells. The extensions diminish with increasing distention of the goblet cell, and the sides of the fully distended "goblet"-shaped cell are smoothed out (Fig 4). Typical terminal bars are seen. The plasma membrane covering the basal cell surface is relatively even, a narrow space separates it from the basement membrane. Luminally, the plasma membrane covers slender, closely packed microvilli. The form of the microvilli changes as the secretory granules accumulate in the luminal part of the cell. They become shorter and broader, until they almost disappear, leaving the cell covered with a smooth, projecting membrane (Fig 5). This finally bursts, discharging the secretory granules on the surface of the epithelium. The secretory granules may coalesce into larger drops of secretion but many, even in the distended goblet cells, remain discrete. They are separated by narrow remnants of cytoplasm. The nucleus is sited in the basal part of the cell. In immature cells it is oval and slightly indented. It is surrounded by an outer and an inner nuclear membrane.

The observations recorded above have been made on goblet cells from both human and rat intestinal mucosa. No species differences were found.

DISCUSSION

The observations presented here have confirmed that the secretory granules of the intestinal goblet cells of the rat develop in relation to a supranuclear, vesicular complex, and have shown that the same applies to the secretory granules in human intestinal goblet cells. The questions now to be answered are: Does this complex represent a cell organelle, and, if so, can it be identified?

The basis for the satisfactory reply to these questions has emerged from the vast literature dealing with the existence and the identity of the Golgi apparatus. Apart from references to reviews in papers by Baker (1957), Lacy & Challice (1957), Dalton & Felix (1957), and Palay (1958), it is beyond the scope of this communication to cite this literature. Suffice it here to state that investigations, first and foremost by Sjostrand & Hanson (1954) and by Dalton & Felix (1954, 1956), have established that the Golgi apparatus is a genuine cell organelle.

ERGASTOPLOSM. It can be distinguished from the vesicles by its specific siting in the cell and by its agranular membranes.

Taking the physiological state of the cells into account the supra nuclear complex of young secreting goblet cells presents just those features characteristic of the Golgi apparatus. The smoothness of the membranes which bound both the flat cisternae and the cisternae resembling secretory granules should be noted in this connection. Consequently, it may be considered as established that the Golgi apparatus plays a part in the mucus production.

It is open to discussion whether it is at all justified to draw conclusions on dynamic processes from morphological studies. Bearing this reservation in mind, the morphological findings do seem, however, to indicate that the mucoid granules are formed with the assistance of the flat cisternae of the Golgi apparatus, which, under gradual dilatation, increase their content of secretory material. The possible part played by the small vesicles has not been determined.

As to the exact nature of the mechanisms involved in the appearance of the secretory material within the Golgi cisternae, morphology provides little information but more ground for speculation. It is not possible to determine morphologically whether the Golgi apparatus is the sole cell organelle that is active in the production of secretion, or whether this is the result of the combined activities of the cell organelles. In the latter case, the accumulation of the secretory material in the Golgi cisternae is only the final stages in the elaboration of secretory granules from products formed elsewhere in the cell. According to this theory, which is widely held and which has found biochemical support, the production of secretion takes place in stages, each stage being effected by one kind of cell organelle in a "Fließbandarbeit", to employ an expression used by *Hirsch* (1960) to express his hypothesis of the production of secretory granules in the exocrine cells of the pancreas. According to current concepts, the ergastoplasmic, dense granules consist mainly of ribonucleic acid (*Palade* 1958), and they are thought to be the sites of the amino acid incorporation in the protein synthesis of the cell. In connection with the close relationship sometimes found between the tubular spaces of the ergastoplasm and the cisternae of the Golgi apparatus, this may indicate a synthesis in the ergastoplasm of secretory material which is then transported to the Golgi apparatus, perhaps for final synthesis there. Assuming such a cooperation and assuming that the synthesis takes place mainly outside the Golgi apparatus, the increasing density of the cisternal contents which accompanies the dilatation of the cisternae, may still indicate that the role of the Golgi apparatus is not merely a passive one. It may be that the membrane of the cisterna participates actively, for instance by effecting a concentration or inspissation of the transported material. Ontogenetic studies of the goblet cell, by correlating the appearance of the cell organelles with the onset of the physiological activity of the cells, may possibly throw further light on the parts played by the different organelles in the production of secretion.

SUMMARY

Mucus production in goblet cells of human and rat jejunal mucosa was examined by electron microscopy. Nearly empty goblet cells from the deep parts of the intestinal crypts presented optimal conditions for the study of the secretory process. Morphological evidence is presented for the origin of mucoid granules in the Golgi apparatus, the secretion accumulating within the cisternae of the apparatus. The goblet cells possess a well developed ergastoplasm. A close relationship has been observed between the ergastoplasm and the Golgi apparatus. The possible collaboration of the ergastoplasm in the secretory production is discussed.

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CHROMOSOME HETEROGENEITY OF CELL LINES IN VITRO

3 *The Effect of Individual Fresh Human Sera on the Mitosis of HeLa Cells*

By

EERO SAKSELA and ERKKI SÄYÄN

Received 1 VIII 61

Numerous authors have reported on the wide variation of the chromosome numbers in all continuous tissue culture cell lines (1, 2, 4, 6, 10). The fact that heteroploid chromosome numbers also characterize cell lines originating from benign tissues, where the extent of heteroploidy is known to be negligible, has raised the question of a presence of a common mechanism of heteroploidization in the tissue culture adaptation of cells. The chromosome number of normal somatic cells applied to tissue culture conditions has been followed from the very first mitoses to the establishment of a continuous tissue culture cell strain (3, 5). A rather clear view of this heteroploid transformation can be obtained on the basis of these studies. First, polyploidization of the cells to the tetraploid stage takes place. In the second phase, numerous anomalous mitoses of these tetraploid cells occur, leading to aneuploid and widely varying chromosome number distribution in the cell culture. In the third phase in this heterogeneous material produced by the chromosome shifts, the cells are able to adapt to the new conditions. The manifold dynamic possibilities of a cell strain, and it might well be thought that the activities displayed by cells here are reflections of more general, adaptive properties of cells facing new environmental conditions.

le of the anomalous mitoses in
to test their possible import-
rather similar shifts in the
cell strain. We have shown
previously that some fresh human sera are capable of changing the
chromosome number distribution of the routine cultivated HeLa cells

Aided by a grant from the Sigrid Juselius Foundation

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(11) The effect of different types of fresh human sera on the general behaviour of HeLa cells also varies, the mode of growth is different and the multiplication rate and the distribution of the nuclear sizes of the cells vary (7,8,9,13) The individual differences of fresh human sera have been found to remain stable in repeated bleedings from the same persons during periods of more than five years. The chromosomal findings are illustrated in Fig 1, presenting the results

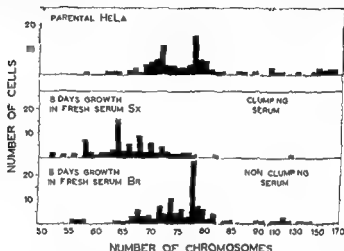


Fig 1

The distribution of the chromosome numbers in the routine cultivated parental HeLa cell strain and in cells cultivated for eight days in fresh human sera of two extreme types Sx and Br

of chromosome counts in one, typical experiment, in which HeLa cells had been cultivated for eight days, by the methods described below, in fresh sera of two extreme types, termed Sx and Br. The disappearance of cells with exceptionally high chromosome numbers and the shift towards lower chromosome numbers in the stem-line region can be observed in the cells cultivated in serum Sx, whereas no such changes are seen in cells grown in serum Br. This feature has been observed in all fresh sera belonging to these two extreme groups studied up to the present.

As it was suspected that such rapid changes in the chromosomal constitution of the cell strain were not due to selection alone but that other adjustive properties also were at work, the possible role of the anomalous mitoses in conjunction with selection seemed worthy of study.

MATERIALS AND METHODS

HeLa cells were used. The routine was to cultivate cells in Roux bottles containing 70 ml of growth medium consisting of 30 per cent heat inactivated (30 in 56°C) and filtrated unselected human serum pool in Hanks solution. For the experimental short term cultures the cell inocula of 20 000 cells/ml were prepared by trypsinization and cultivated in different 30 per cent fresh human sera in Hanks solution in Leibert tubes. The culture medium was changed every day and trypsinization and re-seeding performed after four days of growth.

All the sera used were obtained from the Finnish Red Cross Blood Bank by the courtesy of H. R. Nevanlinna MD.

For determination of the frequencies of the mitotic abnormalities samples from the cultures were taken every second day. Cells growing on the slides were fixed with aceto alcohol and stained with the Feulgen nuclear reaction. The percentage of the anomalous anatelophase figures from all anatelophases counted was determined according to Ietan & Bieseke (5). About 200 mitoses were studied for each determination and the number of normal and abnormal anatelophase figures counted.

RESULTS

Table 1 presents the percentage of anomalous anatelophase figures from all anatelophases (%AA) in the divisions of cells grown in different individual fresh human sera representatives of the two extreme serum groups Sx and Br. The corresponding values of cells cultivated by similar methods in heat inactivated (30 in 56° C) unselected human serum pools are also given. The %AA are given after 2, 4, 6 and 8 days of experimental short term cultivation respectively. Anomalous divisions of frequencies two or three times as high as seen in cells grown in the heat inactivated sera are to be found among cells cultivated in fresh sera. In comparison, the %AA figures of cells grown in different individual fresh sera higher values are found in the group of sera of type Sx. This difference between the two serum groups is also reflected in the mean values of %AA. These are higher among cells grown in the fresh sera of type Sx, the ratio of the mean anomalous anatelophase frequencies in the divisions of cells grown in type Br sera and in type Sx

TABLE 1

The Percentage of Anomalous Anatelophase Figures from all Anatelophases in the Division of Cells Cultivated in Different Human Sera

Serum	Days			
	2	4	6	8
Sx 1	33.9	66.6	40.0	47.3
Sx 2	57.2	54.9	41.5	38.6
Sx 3	57.4	38.9	24.9	15.9
Sx 4	37.2	28.9	31.5	20.0
Mean	44.2	47.3	34.5	31.2
Inactivated 10 1 1	14.7	16.6	16.2	16.8
" 2	15.9	16.4	10.9	6.7
" 3	16.2	15.5	13.7	13.1
Mean	15.6	16.2	13.6	10.5
Br 1	20.0	36.1	21.1	18.8
Br 2	31.8	27.3	24.1	23.4
Br 3	34.8	24.1	23.1	22.4
Br 4	34.4	25.2	21.7	27.9
Br 5	18.7	20.1	20.8	27.0
Mean	27.9	25.8	23.1	23.9

sera varying from 1.13 to 1.18. A graphical illustration of the mean values of %AA in these serum groups is given in Fig 2.

Table 2 shows in detail the frequencies of the different types of the mitotic anomalies observed. The term "malsepindle division" is used to cover a wide variety of spindle deformities ranging from multipolar divisions, where the spindle is so ill-defined that the accurate number of the poles cannot be determined, over increasingly disintegrating

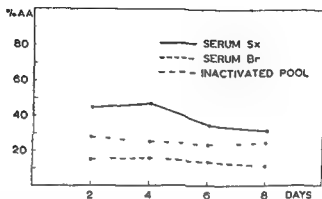


Fig 2

The mean values of anomalous anaphase figures in per cent from all anaphase phases (per cent AA) in the divisions of HeLa cells during 8 days experimental short term cultivation in fresh human sera representatives of the extreme types Sx and Br and in heat inactivated unselected human serum pools.

TABLE 2

The Percentage of Various Types of Anomalous Anaphase Figures from all Anaphases in the Divisions of HeLa Cells Cultivated for 2 and 6 Days in Different Human Sera

Serum	Multipolar spindles		Lagging chromosomes		Bridge formation		Malaligned divisions		Total anaphases		Total mitoses	
	2	6	2	6	2	6	2	6	2	6	2	6
Sx 1	11	12	12	2	7	6	4	20	56	50	250	250
Sx 2	21	10	7	10	6	11	18	10	55	53	226	215
Sx 3	11	7	55	5	8	10	33	17	80	57	218	220
Sx 4	10	11	5	4	11	9	13	8	62	54	250	240
Mean	13	10	7	5	7	9	17	16				
pool 1	3	2	2	4	4	5	5	5	76	68	212	221
pool 2	5			3	3	4	7	5	78	82	141	203
pool 3	2	3	3	3	5	4	11	5	80	72	209	215
Mean	3	2	2	3	4	4	6	5				
Br 1	6	4	8	3	3	7	3	7	35	71	201	223
Br 2	8	6	2	3	5	3	10	10	63	86	203	213
Br 3	10	8	6	2	3	6	15	6	72	78	200	200
Br 4	8	8	4		3	3	20	12	90	97	210	214
Br 5	2	1	2	5	2	8	11	12	80	85	221	223

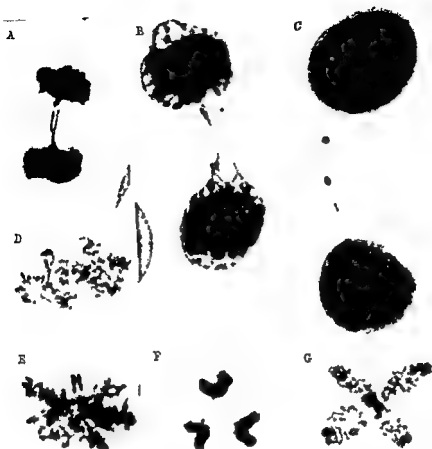


Fig 3

A and B Bridge formation C. Lagging chromosomes
 D and E. Malisindle divisions F Tripolar division G Quadripolar division

spindle apparatus to the clear C mitosis in which each chromosome is free with no traces of the spindle mechanism. Photomicrographs of the various mitotic abnormalities observed are given in Fig 3. The differences in the total %AA figures noted between cells grown in the different serum groups are seen distributed almost similarly among the parts of each individual type of anomaly. All types of the mitotic abnormalities are found more frequently in the divisions of cells grown in fresh sera of type Sx than in type Br. The cells grown in the inactivated serum pools show the lowest number of anomalous mitoses of any type although the differences between the groups of bridge formation and lagging chromosomes and the corresponding values in type Br sera are almost non-existent.

DISCUSSION

The results illustrate that numerous anomalous divisions occur when HeLa cells are cultivated in fresh human sera, and that when the sera have been inactivated by heat, the number of anomalous divisions is "normal" only. Further, it may be concluded that effects of the individual fresh human sera are not all the same, but in some types of sera termed type Sx, the effect is stronger. Human sera of this group have been shown previously to be capable of causing what is termed "clumping" of the cells with a significant growth inhibition, and in addition, to change the distribution of the chromosome numbers of the routine cultivated HeLa cells. These effects are not seen in cells grown in fresh sera of type Br nor in heat-inactivated sera. Since abnormal mitoses result in daughter cells differing in their chromosomal constitution, and also with different possibilities of survival, it may be assumed that the observed differences in effects of the individual fresh human sera on the mitosis of HeLa cells might be of some functional importance in the phenomena mentioned above. Especially the possible role of the abnormal mitoses as a contributory factor of selection in the chromosomal changes observed is stimulating. The occurrence of a new stem-line after 8 days growth in the clumping sera is difficult to be explained as an overgrowth of pre-existing cells of that type alone, but production of new cells of this type from cells with different chromosome numbers can also be expected (12). The observed tendency of some fresh human sera to reduce—"normalize"—the chromosome numbers of the polyploid and aneuploid—"malignant"—HeLa cells and the possible role of the anomalous mitoses in this process offers an interesting opportunity for speculation about the significance of different types of abnormal mitoses seen in cancer tissue. The occurrence of some types of abnormal mitoses might not always be so much a sign of a high malignancy, but occasionally it may be an indication rather of host resistance, i.e. of growth controlling host factors.

SUMMARY

The frequency of abnormal mitoses was studied in HeLa cells cultivated in different, individual fresh human sera. Some of these sera had previously shown the ability to change the growth behaviour, the multiplication rate and the distribution of the chromosome numbers of routine cultivated HeLa cells, others had displayed no such properties. Significantly higher numbers of abnormal divisions were found in the former type of fresh human sera. The possible role of the abnormal mitoses as a contributory factor to selection in the chromosomal changes observed is discussed, as are also the possible implications of the findings in cancer cytology.

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STUDIES OF CALCIFICATION AND BONE FORMATION IN CHONDROSARCOMA

By

CURT LAGERGREN, ÅKE LINDBOM and GUNNAR SODFRBERG

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Many chondrosarcomas contain regions with radiologically visible calcification, which often has a characteristic irregular snow flake appearance. Such regions can be seen in tumours of all degrees of malignancy, but they are most common in highly differentiated ones (Lindbom *et al* 1961). The cartilage in the tumours is divided into lobules of varying size. The connective tissue between these lobules contains most of the vessels of the tumours. The calcification and ossification usually begin at the border with the interlobular tissue (Ichtelstein). The calcification and a secondary bone formation are considered in some cases to be due to precipitation of mineral salts in a degenerated region, which is later converted to bone by metaplasia (Dahlin). The formation of bone is also said to occur by endochondral ossification (Jaffe).

In connection with studies of the vascular pattern of bone tumours of various types and of different degrees of malignancy (Lagergren *et al* 1960, 1961 a, b) examinations have been made by contact microradiography of calcified and ossified regions in chondrosarcomas and of the structure in the sclerotic zone that often surrounds such a tumour.

MATERIAL AND METHOD

This study was performed on specimens of 7 chondrosarcomas in amputated extremities and specimens of skin metastases obtained from a case of chondrosarcoma all of which contained calcified and ossified regions. After fixing in neutral formalin the tumours were sawn into blocks 0.5-1 cm thick which were radiographed. Representative regions were then selected and divided for microradiographic and histologic study. The part intended for microscopic examination was decalcified and the other part was embedded in methylmethacrylate. The blocks were then ground to a thickness of 100-300 microns and microradiographed by the usual technique (Engstrom 1956).

The size and orientation of the mineral salt crystallites precipitated in the regions of calcification and bone formation were examined by x-ray diffraction. The embedded and ground specimens were examined with a Chestley camera (Lagergren 1956).

To ascertain the amount of collagen some of the specimens were examined under the polarization microscope after the methylmethacrylate had been dissolved and the sections decalcified.

A comparison was made between the vascular pattern and the degree of calcification. For examination of the former the vessels of the amputated extremities were filled with contrast medium (Lagergren *et al* 1960). Some of the 0.5-1 cm blocks were radiographed to ascertain the degree of calcification. They were then decalcified and again radiographed to study the filled vessels.

RESULTS

The degree of calcification and bone formation differed widely from one chondrosarcoma to another and within a particular tumour (Fig 1). Some of the tumours consisted largely of cartilage, with no deposits of mineral salts. In other tumours there were cartilage lobules in the periphery of which evidence of calcification was observed (Fig 2). In other areas the calcification was more extensive, and the cartilage cells appeared as radiolucent areas in the otherwise uniformly calcified tumour (Fig 3). In some tumour regions there was completely calcified cartilage containing areas of organized bone. Some of these presented an immature embryonal appearance, but they usually resembled normal bone with haversian systems (Fig 1). In specimens from the skin metastases there were some regions with varying degrees of calcification of the cartilage cells and others with osseous tissue (Fig 4).



Fig 1

Fig 1 Micro-radiogram of a 200 micron section from a chondrosarcoma showing different degrees of calcification and mineral salt deposits.



Fig 2

Fig 3



Fig 3

Fig 3 Microradiogram of a 100 microns section from a region of a chondrosarcoma in which there is extensive calcification. The cartilage cells are seen as radio-lucent (black) areas $\times 270$

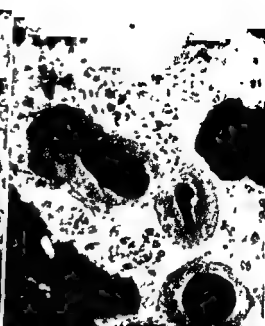


Fig 4

Fig 4 Microradiogram of a 200 microns section from a skin metastasis from a chondrosarcoma. Highly calcified cartilage cells and areas of bone $\times 95$

In the microradiograms from the sclerotic zone surrounding the tumours the sclerotic appearance was due to trabeculae which were thicker and more densely arranged than in normal bone. The bone structure within the trabeculae resembled normal bone in appearance and the density of mineral salts (Fig 5).

The microradiographic structure was in all cases consistent with the histologic structure and was characteristic, so that the tumour structure was easily recognizable.

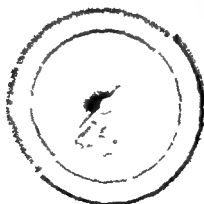
The x ray diffraction study disclosed a difference in the size of the mineral salt crystallites in the various structures (Fig 6). In the cartilage where there was calcification only in the periphery of the lobules the crystallites were larger than elsewhere. The precipitated material in all cases consisted of hydroxyapatite. In normal bone hydroxyapatite crystallites are about 600 Å in length (Carlstrom & Glas 1959) and in the normal dental enamel, which is also composed of hydroxyapatite, they are about 1600 Å (Glas & Omnell 1960). In the slightly calcified cartilage lobules the crystallites were estimated to be about 1000 Å long, whereas in the strongly calcified cartilage and in the osseous tissue in the tumour they were similar in size to those in normal bone (Fig 6).

In bone tissue the apatite crystallites are normally disposed in definite directions, as can be recorded on diffractograms. A similar orientation was found in the regions of the tumours which consisted of bone.



Fig. 5

Micrograph of a 200 micron section from a sclerotic zone round a chondrosarcoma. The trabeculae are thicker and more densely arranged than in normal bone $\times 90$



A

B

Fig. 6

X-ray diffraction pattern of hydroxyapatite from two areas of a chondrosarcoma. A from bone where the inner diffraction ring shows meridional maxima due to the orientation of the crystallites. B from calcified cartilage. There is no orientation of the crystallites. The outer ring is more distinct suggesting that the crystallites are larger.

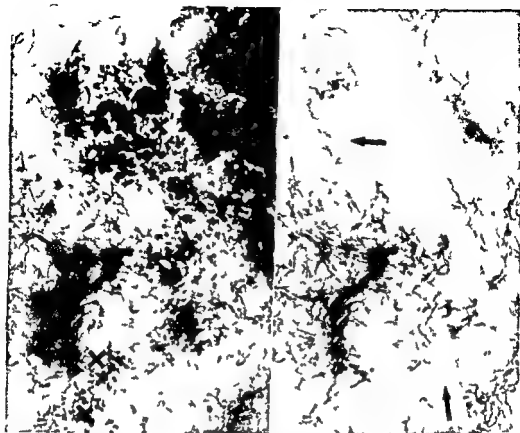


Fig. 7

Radiographs of a 0.5 cm slice of a chondrosarcoma with varying degrees of calcification. The vessels are filled with contrast medium. *A* before *B* after decalcification. The strongly calcified regions (dark) indicated by a \times (in *A*) are seen in *B* to contain fewer vessels than the uncalcified area indicated with arrows (Calcium salt and contrast medium in the vessels are dark the reverse of the microradiogram).

whereas the crystallites in the more or less strongly calcified cartilage displayed no such orderly arrangement.

The examination of decalcified specimens under the polarization microscope showed that in the regions consisting of cartilage there were only streaks of double refracting material consisting of collagen and this was situated in the tissue between the cartilage lobules. In the regions with bone of normal appearance on the other hand such material occurred in quantity and in cross sections of the haversian systems it displayed the concentric stratification typical of normal bone.

Comparison of the vascular picture after decalcification with the pattern of calcification in the non decalcified blocks showed that such regions of the tumour as were most strongly calcified were provided with fewer blood vessels than the uncalcified regions (Fig. 7).

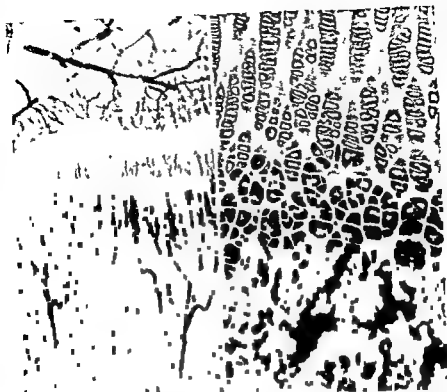


Fig 8

Fig 9

Fig 8 Microangiogram of a zone of growth. The long straight vessels in the metaphysis lead towards the epiphyseal plate at the border of which calcification starts (see Fig 9) $\times 4$

Fig 9 Microradiogram of a 10 microns section of a growth zone. At the top of the figure there is a zone of cartilage proliferation with its characteristic columnar structure. Trabeculae in the lower part of the figure are lined with osteoblasts $\times 130$

DISCUSSION

According to the major text books on bone tumours (Jaffe, Dahlin, Lichtenstein) there are various ways in which bone can be formed in the chondrosarcoma. It can occur in a degenerated or necrotic cartilage, which is calcified primarily. It can also occur in the ground substance between the cartilage lobules. Normally it occurs in zones of growth, whereby there is a continuous process of degeneration, death and calcification of cartilage cells adjacent to the metaphysis. From the metaphysis blood vessels grow and with them osteoblasts, which convert the calcified region to trabeculae (Ham 1952) (Figs 8 and 9). The conditions for this process obtain also in the chondrosarcoma.

In the connective tissue around the cartilage lobules there is a fairly richly developed vascular system, and in certain regions of the tumour



Fig 10

Microradiograph of a 200 microns section of a chondrosarcoma. The arrangement of the cartilage cells is reminiscent of the columnar structure in the zone of growth (in Fig 9) $\times 90$

there are formations of cartilage cells reminiscent of those seen in zones of growth (Fig 10). It is unlikely that the presence of osseous tissue in the chondrosarcoma can be due to the fact that the tumour originates and gradually extends within a bone, as is occasionally suggested for calcified bone and cartilage were also found in metastases remote from bones. It must be inferred therefore that the osseous tissue formed within the chondrosarcoma itself.

In some regions of the chondrosarcoma calcification was present which in radiographs, often had a characteristic snow-flake appearance (Fig 7 a), a pattern produced by cartilage lobules in various stages of calcification (Fig 2). Bone tissue was found only in regions where large coalescent areas of calcification were visualized. The inferior vascular supply in the calcified regions of the tumour suggest that the process of calcification is a result of impaired nutrition and consequent degeneration or necrosis of cartilage tissue (Fig 7). It is interesting to note that the calcification in scleroderma has been attributed to impaired blood supply in the calcified areas.

The x-ray diffraction examination showed that hydroxyapatite was precipitated irregularly in the cartilage, and only in the osseous tissue of the tumour was it organized as in normal bone (Engfeldt 1958).

Examination under the polarization microscope showed that with the re crystallization of the hydroxyapatite in a more regular pattern there was a corresponding formation of the bone matrix. That the hydroxyapatite crystals were longer in the less strongly calcified cartilage than in normal bone tissue has several possible explanations. It has been shown by *Glimcher et al* 1957 that the hydroxyapatite crystals in the bone tissue are precipitated within collagen fibres and it is therefore possible that the collagen serves as centres of crystallization and determines the length of the crystallites. The examination under the polarization microscope showed the presence only of faint streaks of collagen in the cartilage where the crystallites were longer than normal. Any property by which the collagen determines the size could therefore not have been active there. In two cases of scleroderma there was also a variation in crystallite size and a similar variation has been reported in lung calcification (*Lindgren* 1961). The size of the crystallites may also depend on the rate of precipitation. The process of calcification in the chondrosarcoma is often slow whereas in the growth zones it is rapid. The x ray diffraction study of calcified cartilage cells in the growth zone of the growing subject disclosed no difference in crystal size in calcified cartilage cells and in the newly formed trabeculae. In radiographs of chondrosarcomas situated in long bones there is often a thickening of the cortex round the actual tumour. The present microradiographic and histologic examinations showed that the sclerotic zone consisted of ordinary bone formed as a result of the slow expansion of the tumour so that trabeculae were formed which were thicker and more densely arranged than in normal bone. This bone had the lamellar structure of cancellous bone although exhibiting the same density as compact bone (Fig 5). In the sclerotic zone there was no tumour tissue. A sclerotic condensation of bone is seen not only in chondrosarcomas but also in a number of other slowly developing processes in bone such as sclerotic metastases and chronic osteomyelitis.

SUMMARY

A biophysical study has been performed of 8 chondrosarcomas. The characteristic snowflake pattern observed in radiographs of chondrosarcomas is due to calcification of the cartilage lobules. The areas of bone tissue sometimes seen are produced in the tumour tissue. The formation of bone can have several possible explanations. The process may be similar to the one occurring in growth zones or since calcified and ossified regions in the tumours have poorer vascularization than the uncalcified regions the formation of bone may originate in necrotic cartilage. The sclerotic zone which often encloses a chondrosarcoma is due to a thickening of the cortex which consist of normal bone.

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PSEUDO PELGER LEUKOCYTIC ANOMALY IN HAEMATOLOGIC DISORDERS

By

OLLI HEINIVAARA and W. J. KAIPAINEN

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The hereditary Pelger Huet anomaly named after the workers who first described it *Pelger* (1928) and *Huël* (1931) is manifest as a poor segmentation of nuclei and a condensation of nuclear chromatin in granulocytic leukocytes. A sharp contrast between the mature structure and immature shape of the nucleus is typical. The nuclei of anomalous granulocytes are either round band form or bilobed. If all the granulocytes are like this full carriers are in question but if only some of the patient's cells are of this type the others having ordinary multilobulated nuclei they are classified as partial carriers. The heterozygous forms representing round band form or bilobed nuclei are regarded as harmless hereditary anomalies. In the homozygous form all the nuclei are unsegmented. Homozygous animals have shown numerous other anomalies and are generally unfit to survive (*Jonsson et al* 1948, *Nachtsheim* 1950). Three homozygous Pelger Huet cases in human subjects are known (*Haverkamp Begeman & van Lookeren Campagne* 1952, *Bernard & Undrit* 1956, *Slobbe* 1959, *Undrit* 1960).

The presence of a similar defect in nuclear segmentation in the granulocytes in connection with myeloses was noted by *Rohr* (1948) who called the cells pelgeroid paraleukocytes. *Heilmeyer* (1951) and *Undrit* (1954) called them pseudo Pelger cells a term now generally used for cases in which it has not been possible to establish a hereditary genesis of the granulocytic anomaly. A great number of such cases has been reported in the literature.

Table I records all of the Pseudo Pelger cases we have encountered in the literature. It must be emphasized that differentiation between the hereditary Pelger Huet anomaly and a pseudo-Pelger case has not always been precise since information has been inadequate at least in some of the cases. In our opinion however the cases listed in the table justify the classification Pseudo Pelger. In addition to the cases enumerated in the table the literature contains reports also of the occurrence of pseudo Pelger cells in acute myeloid leukaemia (e.g. *schwerner* 1961).

and in connection with bony metastases of malignant tumours and with other cachectic conditions (Undritz & Sepibus 1957). These reports, however, provided no detailed description of the individual cases and hence the cases were not included in the table.

TABLE 1
Pseudo-Pelger Cases Published in the Literature by 1961

Author	Disease
Rohr 1948	Myelogenous leukemia, 6 cases
Heckner 1948	Enteritis, enterocolitis, 6 cases
Dubois-Lericre 1949	Chronic myelogenous leukemia, 1 case
Schoen & Tischendorf 1950	"Darmbrand", 1 case
Flüde 1951	Chronic myelogenous leukemia, 1 case
Heilmeyer 1951	Agranulocytosis, 1 case
Alder & Schaub 1952	Agranulocytosis, 1 case
Daen 1953	Chronic myelogenous leukemia, 1 case
Baltzer 1953	Chronic myelogenous leukemia, 1 case
Degenhardt & Wiedemann 1953	Janconi's panmyelopathy, 1 case
Undritz 1954	Malaria, 1 case
Darte et al 1954	Chronic myelogenous leukemia, 1 case
Böttner & Reinecke 1955	Plasmocytoma, 1 case
Bernard & Undritz 1956	Chronic myelogenous leukemia, 1 case
Dorr & Moloney 1959	Acute myelogenous leukemia, 4 cases; chronic myelogenous leukemia, 6 cases; myeloid metaplasia, 3 cases (including 2 polycythemia); chronic lymphogenous leukemia, 1 case
Heinlaara & Kaipainen 1961	

As will be seen from the table, the pseudo-Pelger phenomenon has been reported most commonly in connection with chronic myelogenous leukemia. The majority of the other pseudo-Pelger cases occurred specifically in connection with haematological diseases. This is an interesting phenomenon in itself and led us to supplement the casuistics by publishing the following pseudo-Pelger cases diagnosed in our clinic. They were all established in connection with haematological diseases. Moreover some of these were diseases not previously reported in connection with the pseudo-Pelger phenomenon.

OWN SERIES

Our cases are reported briefly in Table 2. The first and second types, viz the chronic myelogenous leukemia and aplastic anemia with myelofibrosis, have been discussed before in the literature. But the pseudo-Pelger anomaly has not hitherto been associated with monocytic leukemia, erythremic myelosis and erythroleukemia. The last case, No. 7, a chronic lymphogenous leukemia, was included for the sake of completeness. As seen from Table 1, we have reviewed it earlier in detail.

We feel convinced that all of the cases were pseudo-Pelger conditions. The relatives of 4 patients were examined but no Pelger cells were demonstrated in any of them. In cases (Nos. 4, 5 and 6) where we had

TABLE 2

Cases of Pseudo Pelger Leukocytic Anomaly Percental Distribution of the Neutrophilic Granulocytes Round Band Form and Bilobed Nuclei Show Typical Pseudo Pelger Cells Other Cells show Trilobed Cells and other Granulocytes which Are not Pelger Cells

Patient	Date	Nucleus of neutrophilic granulocytes			
		round %	band form %	bilobed %	other cells %
1 HP 30 yrs female Chronic myelogenous leukemia Treated with myleran since 1955 died March 1960 No Pelger cells observable before November 1958 None of the patient's relatives displayed Pelger cells	14 11 59	11	12	8	69
	22 12 59	30	24	16	30
	24 2 60	49	32	18	8
2 VJ 60 yrs male Aplastic anaemia with myelosclerosis Treated since 1957 with blood transfusions and corticosteroids Pelger cells first observed in October 1958 The relatives displayed no Pelger cells	9 10 58	4	16	22	58
3 ST ■ yrs female Acute monocytic leukemia of myelo monocytic (Naegeli) type Admitted 3 8 1960 corticosteroid treatment died 4 10 1960 The relatives had no Pelger cells	3 8 60	1	3	20	76
	19 8 60	9	10	14	67
	29 8 60	12	21	23	44
	8 9 60	15	18	19	48
	3 10 60	24	32	23	21
4 KL 49 yrs male Acute monocytic leukemia of myelo monocytic (Naegeli) type Admitted 8 4 1961 corticosteroid treatment Still alive The relatives have not been examined	10 4 61	-	-	-	100
	13 4 61	-	-	50	50
	27 4 61	5	-	58	37
	15 5 61	-	8	34	58
5 RL 31 yrs male Acute erythremic myelosis since 1949 polycythemia vera treated with 11 26 mC	26 11 56	1	1	2	96
6 SO 74 yrs female Erythroleukemia Polycythemia vera treated with 12 mC of P ³² in 1950-52 Thereafter anaemia and myelosclerosis which developed into erythroleukemia in 1960 Pelger cells appeared 1960 The relatives have not been examined	14 10 60	14	19	2	74
7 UN 61 yrs male	29 4 59	18	42	23	17

no opportunity of examining the relatives of the patients the latter had not previously displayed symptoms of a presence of Pelger cells which did not appear until in the terminal phase of the disease. Indeed, this applied to all of the cases and provides further evidence that a pseudo Pelger phenomenon was involved and not a hereditary anomaly.

DISCUSSION

Most of the pseudo-Pelger cases reported in the literature have been combined with various forms of myelogenous leukemia. Although the anomaly involved belongs to the granulocytic series, it is interesting that these pseudo-Pelger cells are present also in haematological diseases which do not specifically affect cells of the granulocyte series. Previously we have published a case of the pseudo-Pelger phenomenon combined with *chronic lymphocytic leukemia*. In this paper pseudo Pelger cells were encountered also in *acute erythremic myelosis* in which no features of concomitant myelogenous leukemia were demonstrable.

Pseudo-Pelger cells were demonstrated in a patient with *aplastic anemia* and concomitant *myelosclerosis*. In such cases the development of granulocytes is disturbed as well, as in the case in which polycythemia vera developed into *erythroleukemia*. Here the pseudo-Pelger anomaly was demonstrated when the character of the disease changed. Also in the case of *acute myelomonocytic leukemia*, pseudo-Pelger cells re-appeared in the phase in which the total peripheral blood leukocyte count dropped to normal, the myeloblasts disappeared almost completely, and the monoblasts became the predominating cells.

In the majority of our cases the pseudo Pelger anomaly developed very close to the terminal phase and clinically perhaps should be regarded as unfavourable symptom. Indeed, a pseudo-Pelger anomaly need not necessarily be fatal which fact is proved by our chronic lymphogenous leukemia patient who is still alive two years after the pseudo-Pelger cells were demonstrated.

The fact that pseudo-Pelger cells are encountered more frequently now than they were before is probably due primarily to the fact that greater attention is paid to them. It may also be attributable to the prolonged duration of haematological diseases ascribable to modern therapy allowing time for changes to develop in the blood picture, and consequently also for the formation of pseudo-Pelger cells.

SUMMARY

The report concerns pseudo-Pelger cells demonstrated in connection with monocytic leukemia, erythroleukemia, erythremic myelosis and aplastic anemia with myelosclerosis.

The appearance of pseudo-Pelger cells in the phase in which the

haematological disease changes its character, for instance from myelogenous leukemia to monocytic leukemia, generally in the terminal phase of the disease, is discussed briefly

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INDUCTION OF INTERSTITIAL NEPHRITIS IN RATS FED PHENACETIN AND NAPA (N-ACETYL-*p*-AMINOPHENOL)

By

LENNART ANGERVALL, LEIF LILJEMAN and KNUT LINCOLN

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In recent years various workers have pointed out the increasing abuse of analgesics containing phenacetin and its probable rôle in the development of interstitial nephritis. As possible causes of the renal lesion have been discussed a direct toxic action of phenacetin and its metabolites or of impurities in the commercial preparations, or a damaging effect of chemically altered hemoglobins or of renal tissue hypoxia. In one of these ways or another the kidney may be abnormally susceptible to bacterial infection.

Nearly all phenacetin entering the human organism is metabolized. Most of it is excreted with the urine as conjugated N-acetyl *p*-amino phenol (Napa) and a very small portion converted to *p*-phenetidin (Brodie & Axelrod 1949). Production of *p*-phenetidin has not been demonstrated after administration of Napa. By means of dietary experiment on rabbits and dogs, *Pletscher et al.* (1958) demonstrated that phenacetin and Napa reduced the average life of erythrocytes in approximately the same moderate degree, whilst *p*-phenetidin produced a much greater reduction. These workers found no signs of interstitial nephritis. Nor has it been shown that increased hemolysis is a factor in the causation of interstitial nephritis (*Miescher & Pletscher* 1958). *Harvald et al.* (1960) declared that 4-chloro-acetanilide—a by-product of making phenacetin from 4-nitrochlorobenzene—is a far more potent producer of methemoglobin than phenacetin and that the renal damage may be caused by the effect of abnormal hemoglobins.

Miescher et al. (1958) showed that rabbits given massive phenacetin doses by stomach tube and intravenous inoculations of coli bacteria developed renal lesions of the same type as in acute human pyelonephritis. Neither phenacetin alone nor coli bacteria alone gave rise to inflammatory renal lesions.

The costs of the present investigation were defrayed by grants from *Draco Lunds Farmaceutiska AB-tiebolag*. The technical assistance of Miss *Flyg Berntsson* is highly appreciated.

Sluder *et al* (1958) induced interstitial nephritis in rats by injecting a *Staphylococcus aureus* suspension intravenously and immediately massaging both kidneys under ether "Saridon", consisting mainly of phenacetin, and acetylsalicylic acid increased the severity of the nephritis.

The present investigation was designed to elucidate the effect of a high phenacetin and Napa intake on the susceptibility of albino rats to experimental haematogenous interstitial nephritis.

MATERIALS AND METHODS

Ninety three albino rats of a Wistar strain were divided into four test groups as shown in Table 1. Only 9 male rats are included in the Napa mixture group because 3 escaped by misadventure at a very early stage.

A group of 19 males and 12 females given acetylsalicylic acid was originally included. Owing to hyperphagia—an unforeseen and interesting development—these rats received about 3 times the intended dose of 0.5 g/kg body weight/day. Most of them lost weight, developed massive enteric bleedings and died long before the end of the conditioning period.

TABLE 1
Classification of Experimental Rats

Group	Drug added to food	g/1000 g food	Number of rats			
			Initially		After 21 weeks	
			Males	Females	Males	Females
I	None	—	12	12	12	11
II	Phenacetin	5.3	12	12	12	11
III	Napa	4.55	12	12	12	12
IV	Napa mixture*	1.90	9	12	9	12
Total			45	48	45	46

* 1.60 g Napa + 0.27 caffeine + 0.03 g prothipendyl HCl ("Azacón")

The rats were given tap water and food in the form of a powder of the following composition (Engfeldt 1950) to which the drug was added:

Maize flour	5 kg	Sodium chloride	50 g
Wheat germs	0.5 kg	Potassium chloride	50 g
Wheat gluten	2 kg	Magnesium sulphate	50 g
Casein sodium	1 kg	Ammonium phosphate	115 g
"Fvomlin purum"	1 kg	Ferric citrate	10 g
		Calcium carbonate	200 g

"Fvomlin purum" is a proprietary preparation of vitamin D combined with all the vitamins and growth factors found in yeast. This standard diet contains about equal parts of calcium and phosphorus and the absolute calcium content is about 1 per cent. The vitamin D content is about 50 IU per g food.

The food intake was determined per week for each group. Each rat was weighed once weekly.

All rats surviving after 21 weeks were given a 6-hour meal extract broth culture. The estimated 21 approximately 400 mill kidney was massaged between thumb and wall for 2 minutes. The infection was

ether anesthesia. The rats were then put on the same diet as before and killed with ether on the 11th day after the injection of coli bacteria.

Bacteriological Technique

The *E. coli* strain had been maintained for some years by regular subculture on solid medium and was ordinarily used for assaying antibiotic activity.

In preliminary experiments on rats this strain of coli bacteria gave rise to interstitial nephritis of the massaged left kidney, whilst the unmassaged right kidney remained intact.

The coli strain recovered from an infected rat kidney was subcultured twice and then maintained under paraffin oil in deep agar culture.

Broth inoculated from the deep agar culture was incubated at 37° C for 18 hours and then seeded into fresh sterile meat extract broth, which was incubated at 37° C for 11 hours immediately cooled to 4° C and maintained at that temperature until all the rats were inoculated. Before and after the inoculation of the animals 0.1 ml of serial tenfold dilutions of the broth culture were spread on Drigalski agar plates and the colonies counted on plates with 30-300 colonies.

Histo-pathological Technique

The rats were killed under ether on the 11th day after the injection of coli bacteria. The kidneys were weighed, inspected and prepared for microscopical examination. Hematoxylin, Hansen van Gieson and hematoxylin-eosin were used for staining the histological sections.

The lesions were graded into three groups of increasing severity in accordance with the following scale (cf. Table 2).

- | | | |
|---------|---------|--|
| Grade 1 | (= +) | Occasional comparatively small inflammatory infiltrations occurring predominantly corticomedullary, periglomerularly and perivascularly. |
| Grade 2 | (= ++) | Several, both comparatively small and large, sometimes confluent infiltrations, sometimes solitary cortical infiltrations with fibrosis. |
| Grade 3 | (= +++) | Multiple and confluent infiltrations, often cortical infiltrations with fibrosis. |

RESULTS

According to the manufacturers, the phenacetin and Napa products used in the present investigation were free from 4-chloro-acetanilide. Moreover, no trace of this compound was found in either product when tested by the method described in the 1958 Edition of the British Pharmacopoeia—a procedure capable of determining 4-chloro-acetanilide concentrations of 0.005 per cent.

During the pre-treatment period one rat in the control group and one in the phenacetin group died. Owing to intrathoracic hemorrhage 4 rats died within 10 minutes of the injection of coli bacteria. During the 11 days after the coli injection none of the rats died. Two of the surviving rats were found to have pericarditis.

Weight curves for 45 males and 46 females in the various groups which survived up to the time of the bacterial inoculation are shown in Figs. 1 and 2. There was a tendency to inhibition of weight gain for the males in the phenacetin and Napa groups.

Dietary intake curves have been plotted in Figs. 3 and 4. They disclose no significant deviations between the groups.

TABLE 2
Degree and Distribution of Nephritic Lesions

Group	Drug and dosage		Sex	Sum per of rats	Left Kidney					Right Kidney				
		g kg body weight and day			Normal	+	++	+++	Total	Normal	+	++	+++	Total
I	No drug		m f m+f	12 9 21	2 4 6	4 2 6	4 3 7	2 0 2	10 5 15	11 6 17	1 2 3	0 1 1	0 0 0	1 3 4
II	Phenacetin	0.24 0.40	m f m+f	10 11 21	7 5 12	1 2 3	1 3 4	1 1 2	3 6 9	9 5 14	1 3 6	0 1 1	0 0 0	1 6 7
III	Napa	0.29 0.74	m f m+f	12 12 24	5 6 11	3 2 5	3 4 7	1 0 1	7 6 13	7 8 15	4 3 7	1 1 2	0 0 0	5 4 9
IV	Napa Mixture (amount of Napa)	0.10 0.12	m f m+f	9 12 21	4 2 6	1 5 6	4 4 8	0 1 1	5 10 15	6 6 12	3 6 9	0 0 0	0 0 0	3 6 9
Total			m f m+f	43 44 87	18 17 35	9 11 20	12 14 26	4 2 6	25 27 52	33 25 58	9 16 25	1 3 4	0 0 0	10 19 29

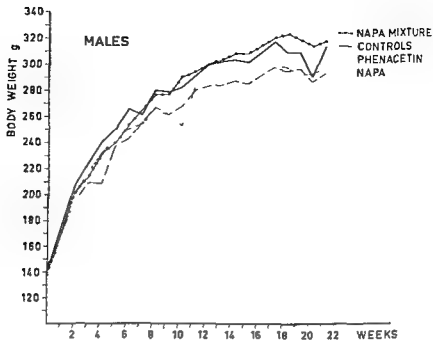


Fig 1
Mean body weight

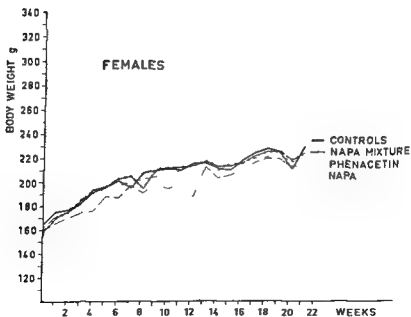


Fig 2
Mean body weight

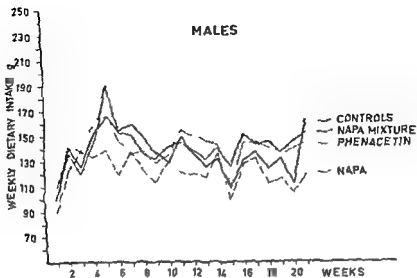


Fig 3
Mean weekly dietary intake

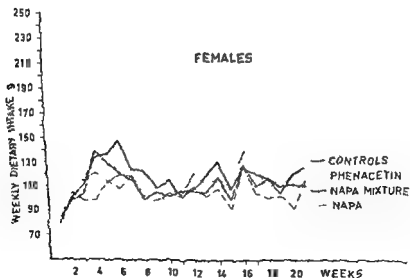


Fig 4
Mean weekly dietary intake

The approximate drug intake calculated on the mean weekly food intake and the mean body weight in the various groups is given in Table 2

Within groups of corresponding sex, body weight and degree of nephritis there were no significant differences in the weight of ipsilateral kidneys between the rats receiving phenacetin and Napa and the control rats

In 52 of all the 87 treated and surviving rats nephritis had developed in the left kidney, and in 29 in the right kidney. A right-sided nephritis did develop only when the left kidney was infected. The degree of severity and distribution of the renal lesions are shown in Table 2

The nephritic lesions were focal and occurred predominantly in the cortex, subcapsularly or along the corticomedullary border but also in the medulla itself. Some lesions extended from the capsule to the renal pelvis, which sometimes also was invaded by inflammatory cells, mostly mononuclears. Many kidneys with interstitial nephritis exhibited gross inflammatory infiltrations, usually multiple, and often subcapsular. Although most of the inflammatory cells were mononuclear a fairly large number were polymorphonuclear. The cortical lesions of some kidneys were fibrotic. Dilated and atrophic tubules contained only leukocytes whilst other tubules also contained a fluid rich in proteins. No necrotic papillae were encountered. Both kidneys from two phenacetin rats exhibited profuse calcified deposits along the corticomedullary border. One of these rats had both kidneys infected, the other rat was not infected.

In all the groups the renal lesions were on the whole graver on the left side than on the right, but the groups did not differ significantly in severity on the same side. Nor was there any difference in severity of the nephritic lesions between males and females.

The incidence of nephritis in either kidney showed a rather wide variation, but there were no significant differences between the groups in testing the hypothesis of independence with the Chi-square test with and without Yates' correction.

Among the rats with left kidney nephritis the incidence of right kidney nephritis was significantly higher in the phenacetin group ($P = 0.021$) and in the Napa group ($P = 0.030$) than in the control group, whereas the incidence in the Napa mixture group did not differ significantly from that in the control group ($P = 0.067$).

The probability values given are calculated by the exact treatment of the two-by-two contingency tables (Fisher 1950).

DISCUSSION

Equimolar amounts of phenacetin and Napa were added to the food. The Napa dose given in the Napa mixture group was about one third of that in the Napa group. The rats in the phenacetin group received ap-

proximately 0.4 g phenacetin daily per kg body weight. A corresponding intake in man would be 20–30 g a day—an excessive dosage.

The admixture of phenacetin or Napa to the food did not effect the dietary intake of the rats. Nevertheless the males in these groups exhibited a weak tendency to inhibition of bodily growth.

Apparently the observed histological changes agreed on the whole with those found earlier in experimental interstitial nephritis (Braude *et al* 1952 Tholen *et al* 1956 Wiescher *et al* 1958 Studer *et al* 1958).

Braude *et al* (1955) introduced the method of giving an intracardial bacterial injection and then immediately massaging the kidneys in order to induce pyelonephritis. They found gross evidence of pyelonephritis in 64 per cent and microscopic evidence in 91 per cent of massaged kidneys and no pathological changes in unmassaged kidneys when using an *E. coli* strain of a certain virulence. With a strain of low virulence no renal inflammation developed within 17 days.

Besides the virulence the size of the bacterial inoculum determines the development of the infection. In experiments on rats with obstructed ureter Gu & Beeson (1956) have shown that reducing the bacterial dose to one tenth (from 200 millions to 20 millions) diminishes the incidence of renal infection by not more than 50 per cent.

A third factor which may influence the incidence and severity of the renal lesions is the mechanical damage exerted by the massage. By massaging the left kidney only the right kidney remains intact and will be influenced apart from the infection mainly by the effect of the drug in the food. The left kidney provides evidence of whether the intracardiac injection has been successful and the bacterial dose adequate.

A dose of 400 million living bacteria is large. That only 60 per cent of the massaged kidneys developed nephritis suggests that the virulence of the adopted coli strain was comparatively low. The laboratory strain of *E. coli* was chosen because it was thought to have attained a certain measure of stability in respect of virulence rate of growth in broth and other properties of importance for reproduction of the experiments.

Figures for the left kidney seem to show that incidence of nephritis is lower in the drug groups than in the control group whereas figures for the right kidney indicate that incidence of nephritis is higher in the drug groups. These contradictory findings may have been caused by imperfections in the injection technique and maybe variations in the dosage of trauma. The development of pericarditis in 2 rats indicates that the injection had been partly extracardiac.

Interstitial nephritis of the right kidney was not encountered in any case unless nephritis was present in the left kidney. Hence the presence of an infectious process in the massaged kidney seems to be necessary for a similar process to develop in the unmassaged kidney.

If the onset of left sided nephritis is regarded correctly the incidence

of rats in each group with left-sided nephritis. Such a comparison shows a significantly higher incidence of nephritis in the right kidney in the phenacetin group and in the Napa group than in the control group.

In contrast to the findings of *Studer et al* (1958) there were no significant differences between the groups in the severity of the renal lesions.

SUMMARY AND CONCLUSIONS

Albino rats, some of which for five months had been given large amounts of phenacetin and Napa, were inoculated intracardially with an *E. coli* broth culture and immediately subjected to massage of the left kidney and killed after 11 days. The incidence and severity of interstitial nephritis in one or both kidneys were assessed and compared between the groups with or without drug intake.

The incidence of right kidney nephritis calculated on the number of rats in each group with left kidney nephritis was significantly higher in the phenacetin and Napa groups than in the control group.

No significant differences in the severity of the renal lesions were found between the groups.

The results suggest that phenacetin and Napa given in excessive doses for a long period promote the development of hematogenous bacterial interstitial nephritis in rats.

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ON ACTION OF NAPA (N-ACETYL-P-AMINOPHENOL) ON THE INDUCTION OF INTERSTITIAL NEPHRITIS IN RATS

By

LENNART ANGERVALL, LEIF LEHMANN and KNUT LINCOLN

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In experiments using albino rats of a Wistar strain Angervall *et al* (1962) made observations suggesting that a regular dietary intake of excessive phenacetin or Napa doses over prolonged periods might promote the development of hematogenic bacterial interstitial nephritis.

The present paper presents the results of an investigation on albino rats of a Sprague Dawley strain designed to elucidate the effect of various Napa doses on the development of interstitial nephritis induced by intravenous inoculation of coli bacteria and massage of one kidney.

MATERIAL AND METHODS

Forty eight female albino Sprague Dawley rats were used in the investigation. They were divided into one control group and two test groups.

After 22 weeks the rats were divided into three groups. The first group was the control group. The second group was the group which received Napa 1.0 g/kg body weight daily for 22 weeks. The third group was the group which received Napa 1.0 g/kg body weight daily for 22 weeks and was then inoculated with coli bacteria. The number of bacteria in the culture was estimated before and after inoculation. The temperature of the rats was estimated before and after inoculation. The number of bacteria in the culture was estimated before and after inoculation. The temperature of the rats was estimated before and after inoculation.

Grade 1 (+) Occasional comparatively small inflammatory infiltrations predominantly occurring corticomedullary, periglomerularly and perivascularly.

Grade 2 (++) Several both comparatively small and large sometimes confluent infiltrations sometimes solitary cortical infiltrations with foci.

Grade 3	(+++)	Multiple and confluent infiltrations, often cortical infiltrations with fibrosis
Grade 4	(++++)	Extremely grave suppurating nephritic lesions with total or subtotal necrosis of renal parenchyma and perinephritis

RESULTS

From the "Napa I" group one rat escaped, one died during the conditioning period, and another died when anesthetized for the injection (autopsy finding pneumonia). The remaining 45 rats survived to the end of the experiment.

All rats developed interstitial nephritis in the left massaged kidney. In the right unmassaged kidney 26 of the rats exhibited nephritic changes (cf. the table). The incidence of interstitial nephritis was the same in the "Napa II" group and in the control group and somewhat higher in the "Napa I" group, but statistically the difference was not significant ($P \approx 0.094$). The probability value is calculated by the exact treatment of the two-by-two contingency table (Fisher 1950). The degree of the lesions was consistently lower in the right than in the left kidney in all animals. Some of the massaged left kidneys were totally or subtotally necrotic and exhibited perinephritic lesions (+++). The degree of the lesions was uniform in the three groups.

Calcifications were encountered in practically all of the kidneys equally distributed on the 3 groups. They were frequently characteristically sited in the cortex close to the cortico-medullary border, and often had the appearance of sparsely scattered, concentrically layered psammoma like grains in tissue without inflammatory changes. Similar small calcium deposits were also present elsewhere in both cortex and medulla. Subcapsular, inflamed regions often contained numerous larger, irregular calcifications.

COMMENTS

The daily Napa dose per kg body weight was approximately 0.4 g in the "Napa I" group and about 0.1 g in the "Napa II" group.

Reckoned by weight, the lower dosage is similar to severe human abuse with daily intake of 5 to 10 g of Napa. The death of 2 rats in the group receiving the higher Napa dosage, as opposed to no death among rats in the group receiving lower dosage and among the controls, may be ascribable to the excessive Napa intake.

The growth curves and dietary intake were essentially the same in all groups. Accordingly the rats exhibited good Napa tolerance even in an excessive dose.

All the 45 rats surviving to the end of the experiments developed interstitial nephritis in the massaged left kidney, in other words the inoculations were 100 per cent successful. Hence, obviously the intravenous technique is superior to the intracardiac route (cf. Braude *et al* 1955, Angervall *et al* 1962).

TABLE
Degree and Distribution of Nephritic Lesions

Drug and dosage	Left kidney		Right kidney										
	g/kg body weight and day	Sum ber of rats	Nephritic					Nephritic					
			+	++	+++	++++	total	+	++	+++	++++	total	
No drug		16	0	0	5	8	3	16	8	3	0	0	8
"Napa I"	0.31	13	0	0	4	7	2	13	3	6	4	0	10
"Napa II"	0.10	16	0	0	3	10	3	16	8	7	1	0	8
Total		45	0	0	12	25	8	45	19	18	8	0	26

The nephritic lesions of the left but not of the right kidneys were more severe in the present experiment than in the previous one. On the other hand the present investigation failed to demonstrate a significant nephritis promoting effect of the Napa intake. These variations in the results of the two experiments may be due to the use of different rat strains or to the modifications in injection technique and infection dose.

The widespread calcifications occurring only in the present experiment may, too, be due to variations between the two rat strains.

The rate of development of calcium deposits being uniform in the Napa and the control groups suggest that Napa *per se* does not noticeably give rise to a development of such depositions. The major cause of the high calcification rates seems to be the high Ca and vitamin D content of the food.

SUMMARY AND CONCLUSION

Albino rats are given intravenous injections of an *E coli* culture and the left kidney is immediately massaged through the intact abdominal wall. The effect of drugs which assumedly influence infection can be evaluated on the basis of incidence and severity of the inflammatory lesions which may develop in the unmassaged right kidney.

One group of female albino rats receiving 0.4 g N-acetyl-*p* amino phenol (Napa) per kg body weight daily for five months, one group receiving one fourth of this dosage, and one control group were treated as described.

The incidence of right kidney interstitial nephritis was higher in the highest Napa dosage group than in the other two groups, but statistically the difference was not significant.

The severity of the renal lesions did not differ in the groups.

The result points in the same direction as our previous finding viz that an excessive Napa intake may promote experimentally induced interstitial nephritis in rats.

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A CONTRIBUTION TO THE BLOOD GROUP FREQUENCIES IN FINNS

By

E. KAARSALO, A. E. KORTEKANGAS, PATRICIA TIPPETT and
JEAN HAMPER

Received 12 vii 61

The distribution among Finns of the ABO, MNS, P and Rh groups has been studied on large numbers of individuals (*Streng* 1935, *Mustakallio* 1937, *Nevanlinna* 1947, *Anttinen* 1953 and *Makela* 1954). How

Many of the tested subjects have married into the large Finnish family, the Ruus which is examined because of the presence in this family of the rare antigen P_k, otherwise subjects have been selected among assistants in a medical laboratory or among members of the blood donor corps. The predominance of persons of group O is ascribable to the donors (47 per cent in our material as against 34.5 per cent in *Mustakallio's* material including 3,156 Finns). The distribution within the groups A, B and AB is about the same as in *Mustakallio's* material.

TABLE 1
The MNS Groups

Anti M anti N anti S anti s sera used		Anti M anti N and anti S sera used	
Cenotype	No. of persons	Phenotype	No. of persons
MS/MS	6	M s	3
MS/Ms	24		
Ms/Ms	19	M s	4
MS/SS	6		
MS/Ss	3	M N s	4
MS/Ss	17		
Ms/Ns	24	M N s	2
SS/Ss	2	N s	0
Ss/Ss	14	N s	1
Total	115		14

The MNSs groups are given in Table 1. The M, MN and N frequencies agree well with the ones reported by *Mustakallio* ($\chi^2 = 0.70$, $df = 2$). The frequencies in the 6 phenotype groups defined by anti M, anti N and anti S agree well with the ones reported by *Makela* ($\chi^2 = 5.74$, $df = 5$). Of all samples anti S agglutinated 50.5 per cent of M samples, 59 per cent of MN samples, 54 per cent and of N samples 12 per cent. Of the 115 samples tested with both anti S and anti s 90 per cent were agglutinated by anti s.

TABLE 2
The P Groups

	Original series			Additional series
	Test cell donors	Married into the P ^k family	Total	
P ₁	72	15	87	406
P ₂	30	12	42	165
Total	102	27	129	571
Percentage of P ₂	29.4	44.5	32.6	93.9

TABLE 3
The Rh Groups

Phenotype	No. of persons	Phenotype	No. of persons
R ₁ r	41	R ₁ ^v R ₁	3
R ₁ R	20	R ₁ ^v R ₂	2
R ₁ R ₁	19	R ₂ R ₂	2
rr	18	R ₁ R ₂	1
R ₂ r	13	R ₁ ^w r	1
R r	8	R ₁ ^u r	1
		Total	129

The frequencies of P₁ and P₂ have been supported by findings from an additional series of 571 unselected donors tested with a view to finding persons in whom the antigen P is absent in the red cells. All the samples were positive with anti P (from P^k persons). In Table 2 the results from the additional material are given separately, as are also findings from individuals involved by marriage in the P^k family. The technique and the antisera used were the same in both series. All P samples from the additional series were confirmed by absorption test at 4° C overnight.

The frequency of P₂ among individuals married into the P^k family seems very high. The additional material consists of 571 unselected individuals who appeared as donors for the first time and whose blood groups were not known in advance. Circumstance divided them into six

groups of about 95 persons, in these groups the percentage of P_2 varied between 23.2 and 40. The rather high percentage of P_2 among the Finns agrees with Anttinen's results, frequencies in his two series were 25.5 and 28 per cent.

The Rh groups are given in Table 3, the following antisera were used: anti D, -C, -c, -E, -e (at least two examples of each), anti C^a and anti f.

Five of the samples were positive both with anti-Lu^a and anti-Lu^b, 124 were negative with anti Lu^a.

Seven of the samples were positive both with anti k and anti-K. 122 were negative against anti-K and all were negative against anti Kp^a.

Samples of group O or A₂ reacted as follows with anti-Le^a and anti-Le^b (two examples of each antiserum)

Le(a+b—)	16
Le(a—b+)	57
Le(a—b—)	3
Total	76

A further 51 A₁ and B samples gave the following reaction with anti-Le^a

Le(a+)	3
Le(a—)	48

The two samples required to make a total of 129 gave doubtful results.

The distribution of the Duffy and Kidd groups are given in Table 4. On 29 of the samples no Kidd tests were made.

TABLE 4
The Duffy and Kidd Groups

Tested only with anti Fy ^a		Tested with anti Fy ^a and anti Fy ^b		No. of persons tested
Fy(a+)	7	Fy(a+b—)	27	
		Fy(a+b+)	53	
Fy(a—)	11	Fy(a—b+)	31	
	18		111	129
Tested only with anti Jk ^a		Tested with anti Jk ^a and anti-Jk ^b		No. of persons tested
Jk(a+)	6	Jk(a+b—)	27	
		Jk(a+b+)	45	
Jk(a—)	2	Jk(a—b+)	20	
	8		92	100

SUMMARY

The distribution of the MNSs, P, Rh, Lutheran, Kell, Lewis, Duffy and Kidd blood groups in 129 Finns not individually related, is recorded. The P groups of an additional 571 unrelated Finns are also recorded.

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DEVELOPMENT OF TUBERCULIN SENSITIVITY AND ACQUIRED RESISTANCE TO TUBERCULOSIS IN GUINEA PIGS VACCINATED WITH A SMALL DOSE OF BCG VACCINE

By

A JESPERSEN, M WEIS BENTZON and M MAGNUSSEN

Received 2 viii 61

A previous study (1) dealt with the measurement of tuberculin sensitivity and acquired immunity in guinea pigs vaccinated with varying doses (10^{-6} to 10^{-1} mg) of BCG vaccine. The examinations were carried out six to seven weeks after vaccination. Under the experimental conditions of that study, tuberculin sensitivity was always accompanied by immunity and there was no difference between the survival times of the tuberculin negative animals in the vaccinated groups and the non vaccinated controls. However, there was no direct relationship between the size of the tuberculin reactions and the degree of acquired resistance after administration of a specified dose. A dose of 10^{-2} mg BCG could induce tuberculin sensitivity and acquired resistance in about 50 per cent of the animals. Though the survival time was prolonged by use of increasing doses of vaccine, there was no pronounced difference in the degree of acquired resistance between the 10^{-1} group and the tuberculin positive animals in the 10^{-2} group.

The aim of the present study was to elucidate further the relationship between tuberculin sensitivity and acquired resistance to tuberculous infection developing in guinea pigs after vaccination with small doses of BCG vaccine. Groups of guinea pigs were vaccinated with 10^{-6} mg or 10^{-2} mg BCG vaccine and the tuberculin sensitivity and acquired resistance measured at periods varying from two to twelve weeks after vaccination.

A subsequent paper (3) reports a study in which the degree of acquired resistance and the tuberculin sensitivity induced by vaccination with a small (10^{-6} mg) and a large dose (10^{-1} mg) is compared, using varying vaccination periods up to nine months.

EXPERIMENTAL

The experiment comprised six groups of guinea pigs with 44 to 48 animals in each group. One half were vaccinated subcutaneously with 10^{-6} mg and the other half with 10^{-2} mg BCG vaccine. The vaccination was carried out at such times (see Table

SUMMARY

The distribution of the MNSs, P Rh Lutheran, Kell, Lewis Duffy and Kidd blood groups in 129 Finns not individually related is recorded. The P groups of an additional 571 unrelated Finns are also recorded.

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- 1 Anttinen E E Occurrence of blood group P in Finland *Ann med exper et biol Fenniae* 31 285 290 1953
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DEVELOPMENT OF TUBERCULIN SENSITIVITY AND ACQUIRED RESISTANCE TO TUBERCULOSIS IN GUINEA PIGS VACCINATED WITH A SMALL DOSE OF BCG VACCINE

By

A. JENSEN, M. WEISBENTZON and M. MACALUSSEN

Received 2 June 61

A previous study (1) dealt with the measurement of tuberculin sensitivity and acquired immunity in guinea pigs vaccinated with varying doses (10^{-6} to 10^{-1} mg) of BCG vaccine. The examinations were carried out six to seven weeks after vaccination. Under the experimental conditions of that study, tuberculin sensitivity was always accompanied by immunity and there was no difference between the survival times of the tuberculin negative animals in the vaccinated groups and the non-vaccinated controls. However, there was no direct relationship between the size of the tuberculin reactions and the degree of acquired resistance after administration of a specified dose. A dose of 10^{-5} mg BCG could induce tuberculin sensitivity and acquired resistance in about 50 per cent of the animals. Though the survival time was prolonged by use of increasing doses of vaccine, there was no pronounced difference in the degree of acquired resistance between the 10^{-1} group and the tuberculin positive animals in the 10^{-6} group.

The aim of the present study was to elucidate further the relationship between tuberculin sensitivity and acquired resistance to tuberculous infection developing in guinea pigs after vaccination with small doses of BCG vaccine. Groups of guinea pigs were vaccinated with 10^{-6} mg or 10^{-1} mg BCG vaccine and the tuberculin sensitivity and acquired resistance measured at periods varying from two to twelve weeks after vaccination.

A subsequent paper (2) reports a study in which the degree of acquired resistance and the tuberculin sensitivity induced by vaccination with a small (10^{-5} mg) and a large dose (10^{-1} mg) is compared using varying vaccination periods up to nine months.

EXPERIMENTAL

The experiment comprised six groups of guinea pigs with 44 to 48 animals in each group. One half were vaccinated subcutaneously with 10^{-5} mg and the other half with 10^{-6} mg BCG vaccine. The vaccination was carried out at such times (see Table

1) that the vaccination time for the individual groups on day of challenge varied from two to twelve weeks with 14 day intervals. Ten days after the last vaccination all the animals and two groups of non vaccinated animals were given intradermal tuberculin tests. Three days later all the animals were challenged with a small dose of virulent tubercle bacilli given intravenously. The animals were observed until they died of the tuberculous infection and the survival time in days after challenge was used as indication of the acquired resistance.

TABLE 1
Guinea Pig Vaccination Schedule

Vaccination period	Vaccination date	No. of animals vaccinated*		BCG vaccine			Vaccination dose $\frac{1}{2}$ (mg) (viable units)	
		Vaccination dose (mg)		SSI lot no	Colony counts (viable units per 0.75 ml)		10 ⁻⁶	10 ⁻⁵
		10 ⁻⁶	10 ⁻⁵		Before vaccination	After vaccination		
■ weeks	15.8.1956	22	22	1218 IA	1.0×10^7	0.7×10^7	11	110
4	1.8.1956	22	22	1216 IA	1.1×10^7	1.1×10^7	14	140
6	18.7.1956	22	22	1214 IA	0.9×10^7	0.7×10^7	10	100
8	4.7.1956	22	22	1212 IA	0.8×10^7	0.7×10^7	10	100
10	20.6.1956	24	24†	1210 IA	1.0×10^7	0.6×10^7	11	110
12	6.6.1956	24	24	1208 IA	1.5×10^7 *	1.5×10^7 *	20*	200*

* Subcutaneous injection

§ Estimated from means of colony counts before and after vaccination

† To compensate for the natural mortality in the vaccination period the groups were larger for the longer vaccination periods

* Approximately

MATERIAL AND METHODS

Animals The animals used were albino guinea pigs of both sexes bred at the farm attached to Statens Seruminstitut. At the commencement of the study 12 before vaccination all the animals weighed 250 to 300 g. They were fed on hay, corn mixture and sugar beet or kohlrabi. Two guinea pigs were kept in each cage.

At the time of challenge the first vaccinated guinea pigs weighed from 500 to 550 g. Two non vaccinated control groups were therefore included in the study: group 1 weighing from 250 to 300 g and group 2 from 500 to 550 g.

None of the guinea pigs showed any reaction to a pre vaccination tuberculin test with 250 tuberculin units (TU).

Vaccination with BCG vaccine Freshly prepared liquid BCG vaccine from the BCG

per n was

water) 1 Each animal was injected subcutaneously in the right inguinal region with 0.2 ml vaccine. The vaccine was protected against light during preparation and use and the vaccination was carried out by artificial light.

Colony counts of the vaccines were made by inoculation of 0.1 ml vaccine on to each of ten tubes of Lowenstein Jensen medium using samples of freshly prepared vaccine (before vaccination) and vaccine exposed to the same conditions as the vaccine used for vaccination (after vaccination). There were fairly small but

vaccination periods of four and twelve weeks, where the doses were slightly larger (15-20 and 150-200 viable units) ²

Tuberculin testing In order to determine the tuberculin sensitivity, the animals were given two intradermal injections of 0.1 ml, one on each side of the back, using Purified Tuberculin Batch RT 22, Statens Seruminstitut. The dose was 250 TU (3.3 µg) per 0.1 ml and the diluent phosphate buffered saline with 0.01 per cent chinosol.

As mentioned above tuberculin injections were given ten days after the last vaccination with BCG vaccine. The reactions were read after 48 hours by two independent readers. The animals were taken cage wise in random order, the readers having no knowledge of the group to which these belonged. The mean value of two diameters of erythema at right angles to each other was recorded. The results given are the averages for the two readers, using the mean of the two reactions of each animal.

Challenge Three days after tuberculin testing the guinea pigs were challenged

medium showed that the suspension contained approximately 1500 bacterial units per ml.

Each animal was injected intravenously into an ear vein with 0.2 ml bacterial suspension i.e. about 300 viable bacterial units. The animals were taken cage wise in random order, and the challenge process took six hours to perform. The bacterial suspension was protected against daylight during preparation and use. Bacterial counts in the suspension were 10⁶ per ml.

tuberculous

Tub 0	Organs normal, culture negative
Tub I	No definite specific changes, culture positive
Tub II	Small tuberculous lesions, mainly regressive
Tub III	Slight generalized progressive tuberculosis
Tub IV	Moderate generalized tuberculosis
Tub V	Severe generalized tuberculosis

The survival times from day of challenge for the animals with Tub IV and Tub V were used as indication of the acquired resistance.

RESULTS

The tuberculin reactions and survival times of the individual animals are given in Appendix Tables 1 and 2. The animals are arranged according to increasing survival times within the groups.

Tuberculin Sensitivity

The distributions of the tuberculin reactions of the non vaccinated animals and of animals vaccinated with 10⁶ mg BCG are shown in Fig. 1. Very few animals had developed any strong tuberculin sensitivity six weeks after the vaccination, but none the less the relative number of zero reactions deviates significantly from the control groups.

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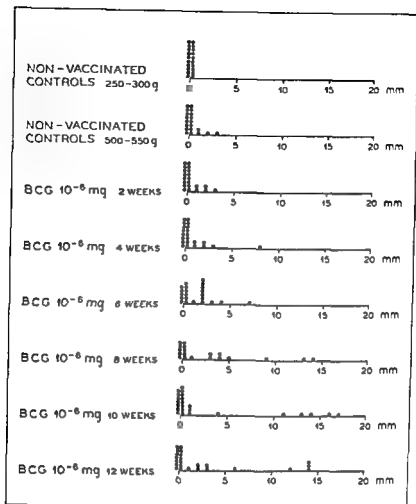


Fig 1

Distribution of tuberculin reactions (erythema in mm) to 250 TU of non-vaccinated guinea pigs and guinea pigs vaccinated subcutaneously with 10^{-6} mg BCG vaccine at specified periods before testing

(50 per cent as against 90 per cent) Ten to twelve weeks after vaccination about 15 per cent of the vaccinated animals had a reaction of from 11 to 17 mm

Four to six weeks after vaccination with 10^{-6} mg BCG vaccine (Fig 2) approximately 50 per cent of the animals had tuberculin reactions measuring 4 to 15 mm. The tuberculin sensitivity increased and ten to twelve weeks after vaccination about 80 per cent of the animals had reactions measuring from 8 to 21 mm. These observations indicate that the tuberculin sensitivity of the animals was still increasing eight to ten weeks after vaccination with 10^{-6} mg BCG.

The fact that after twelve weeks all animals, except one, were tuberculin positive is probably due to the dose of vaccine used being about twice as high as in the other groups.

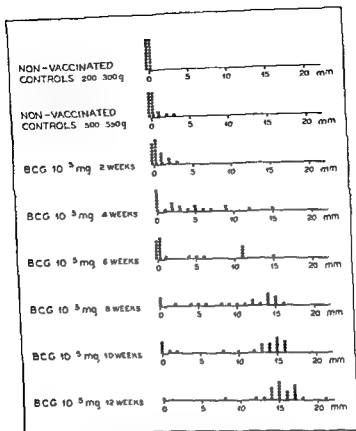


Fig. 2

Distribution of tuberculin reactions (erythema in mm) to 250 TU of non vaccinated guinea pigs and guinea pigs vaccinated subcutaneously with 10^{-5} mg BCG vaccine at specified periods before testing

Survival Times

The majority of the *non-vaccinated controls* died 75 to 175 days ($2\frac{1}{2}$ to 6 months) after challenge (see Appendix Table 1)

The majority of the animals vaccinated with 10^{-5} mg BCG died from 80 to 200 days after challenge (see Appendix Table 1). There is no obvious difference in survival time between these animals and the controls.

Animals vaccinated with 10^{-5} mg BCG four to twelve weeks before challenge died 100 to 250 days (3 to 8 months) after challenge (see Appendix Table 2)

A statistical evaluation of the survival times of the various groups is given in Table 2. The arithmetic mean of the reciprocal survival time (\bar{y}), the standard deviation (s_y), and the median survival time in days ($\frac{1}{\bar{y}}$) are shown for each group.

TABLE 2

Median Survival Time (in Days) after Challenge with Virulent Tubercle Bacilli of Groups of Non Vaccinated Guinea Pigs and Guinea Pigs Vaccinated with BCG Vaccine at Varying Intervals before Challenge

Group		No of animals n	Arithmetic mean of reciprocal survival times $\frac{1}{\bar{y}}$	Standard deviation s_y	Median survival time (in days) $\frac{1}{\bar{y}}$
Vaccination dose	Vaccination period				
Non-vacc	controls 1	20	0.0087	0.0084	116
Non-vacc	controls 2	19	0.0081		
10 ⁶ mg BCG	2 weeks	21	0.0089	0.0021†	113
	4 "	22	0.0076	0.0023†	132
	6 "	22	0.0076	0.0024†	132
	8 "	19	0.0076	0.0023†	131
	10 "	24	0.0085	0.0026†	117
	12 "	23	0.0077	0.0021†	130
10 ⁵ mg BCG	2 weeks	22	0.0088	0.0020	114
	4 "	22	0.0072	0.0020	138
	6 "	22	0.0072	0.0019	138
	8 "	22	0.0066**	0.0018	151
	10 "	24	0.0067**	0.0015*	148
	12 "	24	0.0054***	0.0016*	184

† The estimates of the variances of y found for the different groups are not significantly different. The average for all groups is

$$s_y^2 = 0.00000547 \quad s_y = 0.0023 \quad \sigma_1 = \sigma_y \sqrt{\frac{1}{n}}$$

*, ** and *** denotes that the deviation from the control group is significant 5% > P > 1%, 1% > P > 0.1% and 0.1% > P respectively

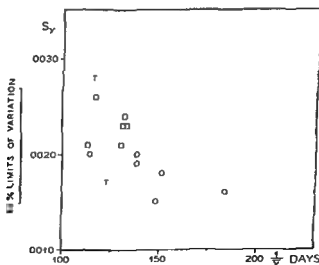


Fig. 3

Relationship between standard deviation of reciprocal survival times and median survival times for groups of non vaccinated guinea pigs (T) and guinea pigs vaccinated subcutaneously with 10⁶ mg (□) or 10⁵ mg (○) BCG vaccine

In Fig 3 the standard deviations are plotted against the median survival time. The figure indicates that the standard deviation decreases with increasing median survival time. A preliminary analysis of the data revealed no significant difference between the control groups and the groups vaccinated with 10^{-6} mg BCG. Therefore an estimate of the standard deviation is calculated by pooling the results for these groups. The value obtained, $s_s = 0.0023$, is used for comparison between controls and vaccinated animals.

The median survival times of the two non-vaccinated control groups show no significant variation, and therefore in subsequent evaluations the two groups are taken together, giving a median survival time of 119 days.

None of the groups vaccinated with 10^{-6} mg BCG deviated significantly from the control groups, and neither could any difference from the control group be demonstrated for the three groups with the longest vaccination time taken together. However, the fact that the median survival time in four of the six groups is slightly longer than that of the non-vaccinated controls indicates that there may be a slight immunizing effect from vaccination with 10^{-6} mg BCG also quite shortly after the vaccination, but the survival time is apparently not affected by prolongation of the vaccination time.

TABLE 3

Median Survival Time (in Days) after Challenge with Virulent Tubercle Bacilli of Groups of Non-vaccinated Guinea Pigs and Guinea Pigs Vaccinated with BCG Vaccine According to Size of Tuberculin Reaction at Time of Challenge

Group	Tuberculin reaction (TR) in mm									
	TR = 0		0 < TR ≤ 20		20 < TR ≤ 50		50 < TR ≤ 110		110 < TR	
	No of ani mals n	Median survi val time (in days) $\frac{1}{v}$	No of ani mals n	Median survi val time (in days) $\frac{1}{v}$	No of ani mals n	Median survi val time (in days) $\frac{1}{v}$	No of ani mals n	Median survi val time (in days) $\frac{1}{v}$	No of ani mals n	Median survi val time (in days) $\frac{1}{v}$
Non-vacc. controls 1	35	118	2	152	2	110	0	—	0	—
Non-vacc. controls 2	—	—	—	—	—	—	—	—	—	—
10^{-6} mg BCG	2 weeks	16	110	3	135	2	105	0	0	—
	4 "	16	133	4	133	1	114	1	123	0
	6 "	11	149	5	120	5	109	1	192	0
	8 "	10	122	1	196	5	152	1	159	2
	10 "	15	118	3	87	1	147	1	164	4
	12 "	14	139	2	130	3	97	1	147	3
10^{-8} mg BCG	2 weeks	15	109	6	123	1	159	0	0	—
	4 "	7	118	4	139	0	169	4	185	2
	6 "	11	141	1	110	2	119	0	143	4
	8 "	3	185	1	182	2	151	0	159	11
	10 "	4	141	2	147	0	—	2	106	16
	12 "	1	373	0	—	0	—	1	250	22

As regards the groups vaccinated with 10^5 mg BCG, the survival time is slightly prolonged from the fourth week and a significant prolongation could be demonstrated eight weeks after vaccination. The median survival time was longer the longer the interval after vaccination. However, it is possible that the higher median survival time twelve weeks after vaccination is explained by the fact that the vaccine dose was about twice as big for this group.

When the results after vaccination with 10^6 mg and 10^5 mg are compared, it can be seen that there is a significant difference only for the groups challenged ten to twelve weeks after vaccination.

Relationship between Tuberculin Sensitivity and Survival Time

The relationship between the tuberculin sensitivity and survival time can be seen from Table 3, which shows the median survival time of groups of guinea pigs according to size of tuberculin reactions at time of challenge.

The median survival time of the 14 animals vaccinated with 10^6 mg BCG vaccine with tuberculin reactions ≥ 5 mm is 138 days. This value shows a deviation from that of the non-vaccinated controls of 1.58 times the standard error.¹ This is not statistically significant. On the other hand, the survival times for these animals do not deviate significantly from those of the animals with corresponding tuberculin reactions vaccinated with 10^5 mg BCG ($\frac{1}{y} = 156$), whereas the deviation between this median survival time and that of the controls is statistically significant.²

The median survival time of the eight animals which still had TR = 0 mm eight to twelve weeks after vaccination with 10^5 mg BCG is 167 days. This deviates significantly from the median survival time of the controls and the animals vaccinated with 10^6 mg BCG TR = 0 mm.³

No relationship could be demonstrated between the tuberculin sensitivity and the survival time *within* any of the groups.

DISCUSSION

In the present work, as in a previous study (1), the tuberculin reaction of many of the controls and of some of the vaccinated animals is recorded as 0 mm, while in three other previous experiments (2, 3, 4)

¹ $y = 0.00724$ $n = 14$ Deviation from control = 0.00115 SE = 0.00073

² $y = 0.00639$ $n = 69$ Deviation from control = 0.00200 SE = 0.00047

³ 10^5 mg $y = 0.00595$ $n = 8$ 10^6 mg $y = 0.00793$ $n = 39$ $\Delta = 0.00198$

SF = 0.0023 $\sqrt{\frac{1}{8} + \frac{1}{33}} = 0.00091$ (The value used for the standard deviation

$s_y = 0.0023$ is possibly too high in this comparison since the standard deviation is lower for the eight animals vaccinated with 10^5 mg see Table II results 8-12 weeks)

a tuberculin reaction is seldom registered as 0 mm. This is not due to any actual difference in the reactions of the animals, but because in the first mentioned experiments the reactions were read after 48 hours as against 24 hours in the other works. Generally the size of the tuberculin reaction of guinea pigs is smaller after 48 hours than after 24 hours particularly in the case of small reactions. In addition a slight difference in the reading technique in the two experimental groups is also of significance.

The study has shown that 10^{-6} mg BCG (10 to 20 bacterial units counted by culture on Löwenstein-Jensen medium) injected subcutaneously is too small a dose to induce a definite sensitizing or immunizing effect in guinea pigs. A slight tuberculin sensitivity can be demonstrated already after six weeks but after three months only 5 out of 24 animals had a reaction larger than 5 mm. Since the number of animals with positive tuberculin reaction was not larger after 12 weeks than after 8 and 10 weeks it can be assumed that the number would not become increased were the vaccination time longer than three months.

No significant immunity could be demonstrated after vaccination with 10^{-6} mg with the experimental technique used but a slight prolongation of the survival time could be seen for animals vaccinated at least four weeks before challenge. In addition 14 animals with tuberculin reactions ≥ 5 mm had a median survival time of 138 days as against 119 days in the control groups.

A dose of 10⁻⁶ mg BCG (100 to 200 bacterial units) had a strong effect. Tuberculin sensitivity was demonstrable after four weeks and after three months all the animals except one were tuberculin positive. The effect of vaccination in this dose range was highly influenced by variations in the dose of BCG. After four and twelve weeks the dose was larger than in the other groups and here a relatively larger tuberculin sensitivity was observed.

As in the 10^{-6} group the survival time was slightly prolonged from the fourth week. From the eighth week the prolongation was significant and after three months it was 184 days as against 119 days for the controls. Thus the present study indicates that the minimum dose of BCG vaccine which will cause all—or almost all—the guinea pigs in a group to become tuberculin positive and which will evoke an acquired resistance in the animals after subcutaneous vaccination is in the neighborhood of 200 bacterial units. Tønderlund *et al.* (1) examined the development of tuberculin sensitivity in guinea pigs vaccinated intracutaneously with various doses of BCG vaccine and found a similar result as regards tuberculin sensitivity. However the immunity was not measured in that study. In another previous study (2) where the vaccination period was also three months a BCG vaccine containing about 60 viable units induced a tuberculin reaction (and immunity) in two thirds of the animals whilst a ten times stronger suspension caused all the guinea pigs to become tuberculin positive (and immune). In that

study the vaccine was grown on Dubos medium and the animals were vaccinated intracutaneously

It was observed in previous works that of animals vaccinated with 10^{-2} mg BCG (1, 3) or with about 60 bacterial units (2), only those with a certain tuberculin sensitivity at time of challenge had acquired immunity. As mentioned, a significant prolongation of the survival time was found in the present study in eight animals vaccinated with 10^{-1} mg eight to twelve weeks before challenge which had no increased tuberculin sensitivity at time of challenge

There are several possible explanations for the apparently different results in the present study. It might be that some of these "negative" animals had weak tuberculin reactions 24 hours after injection, but that these had disappeared by the time of reading (after 48 hours)

It might also be that both tuberculin sensitivity and acquired resistance were in course of development in these animals at the time of challenge and that they would have become tuberculin positive shortly after challenge. It is reasonable to assume that the acquired resistance thus developing would manifest itself in the form of a prolonged survival time in the animals in question. However, it seems more likely that these animals would never become tuberculin positive. Ten and twelve weeks after vaccination there were very few animals with small tuberculin reactions, i.e. animals whose tuberculin sensitivity could be assumed to be in course of development. The fact that after twelve weeks there was only one negative animal is, as mentioned previously, probably due to the dose of vaccine having been twice as high.

If neither of these reasons applies in the present study, there is a group of eight animals which had acquired resistance after BCG vaccination but were not tuberculin positive. On the basis of the information existing at present it is not possible to exclude that this is actually the case. At any rate, it is remarkable that no such group was seen in three other studies (1, 2, 3).

Determination of the acquired resistance by means of survival time must necessarily stretch over a long period, and is influenced, in addition to the interaction between the infecting bacteria and the immune host organism, by intercurrent disease and by the diet and care of the animals. On the other hand tuberculin sensitivity, both in the present work and in the majority of the previous studies is measured at a given time (time of challenge). However, the tuberculin sensitivity changes with time during the infection period not only as the result of the challenge but also in relation to the vaccination time, dose of vaccine, method of vaccination, etc., and one single estimation of the tuberculin sensitivity at time of challenge cannot be said to give an indication of these latent elements.

It must also be remembered that, at any rate in some cases, the tuberculin sensitivity can be changed by the tuberculin test itself, either

in the form of an increase or a decrease dependent on the size of the tuberculin dose the level of tuberculin sensitivity duration of vaccination period etc

Taking into consideration the complexity and variability of the two characteristics being measured i.e. acquired resistance and tuberculin sensitivity it cannot be surprising that varying degrees of relationship between the two aspects have been found in the different studies Very comprehensive animal experiments carried out under varying experimental conditions are necessary before it is known whether the degree of tuberculin sensitivity can be used as indication of the acquired immunity induced by vaccination with BCG vaccine

SUMMARY

The study includes six groups of guinea pigs with 44-48 animals per group Half the animals were vaccinated subcutaneously with 10^{-6} mg BCG vaccine and the other half with 10^{-4} mg Vaccination was carried out at such times that the vaccination times for the six groups on the day of challenge were 2 4 6 8 10 and 12 weeks respectively Ten days after the last vaccination the animals and two groups of non vaccinated controls were tuberculin tested Three days later all the animals were challenged intravenously with 10^{-6} mg virulent human tubercle bacilli They were observed until they died of the tuberculous infection The survival time in days after challenge was used as indication of the resistance acquired

The guinea pigs vaccinated with 10^{-4} mg BCG vaccine developed slight tuberculin sensitivity in a few cases only Even three months after vaccination no significant prolongation of the survival time could be demonstrated

However 10^{-6} mg BCG (100-200 bacterial units) had a strong effect Three months after vaccination 23 out of 24 animals were tuberculin positive and the median survival time was 184 days as against 119 for the controls

It was not possible in the present work to demonstrate as close a relationship between tuberculin sensitivity and acquired resistance in guinea pigs vaccinated with a small dose of BCG vaccine as in previous studies

APPENDIX TABLE 1

Survival Time (in Days) after Challenge with Virulent Tubercle Bacilli and Size of Tuberculin Reaction (in mm) to 250 TU at Time of Challenge of Non-vaccinated Guinea Pigs and Guinea Pigs vaccinated with 10 mg BCG Vaccine at Specified Intervals before Challenge

Order of survival	Non vaccinated controls										Vaccination period									
	Gr 1 wt 200-300 g					Gr 2 wt 500-500g					2 weeks		4 weeks		6 weeks		8 weeks		10 weeks	
	Tuberculin reaction	Survival time	Tuberculin reaction	Survival time	Tuberculin reaction	Tuberculin reaction	Survival time	Tuberculin reaction	Survival time	Tuberculin reaction	Tuberculin reaction	Survival time	Tuberculin reaction	Survival time	Tuberculin reaction	Survival time	Tuberculin reaction	Survival time	Tuberculin reaction	Survival time
1	0	76	0	35*	0	78	0	75	0	87	0	79	0	79	0	87	0	79	0	87
2	0	77	0	91	0	80	0	106	0	92	0	96	0	96	0	92	0	96	0	92
3	0	77	0	92	0	91	0	106	0	96	0	102	0	102	0	96	0	102	0	96
4	0	83	0	99	0	91	0	107	0	96	0	102	0	102	0	96	0	102	0	96
5	0	100	2.0	102	0	97	1.0	110	1.0	98	0	106	1.0	106	1.0	98	0	106	1.0	98
6	0	91	0	109	0	99	0	114	0	99	0	107	0	107	0	99	0	107	0	99
7	0	95	0	113	0	100	0	117	0	104	0	113	0	113	0	104	0	113	0	104
8	0	99	0	115	0	104	0	123	0	116	0	123	0	116	0	116	0	123	0	116
9	0	117	0	119	0	104	0	124	0	116	0	124	0	116	0	116	0	124	0	116
10	0	119	0	120	0	106	0	127	0	121	0	132	0	121	0	121	0	132	0	121
11	0	140	0.5	121	0	110	1.0	130	0	127	0	137	0	127	0	127	0	137	0	127
12	0	142	(2.5)	121	0	127	0	132	0	131	0	147	0	131	0	131	0	147	0	131
13	0	151	0	125	0	127	0	133	0	132	0	147	0	132	0	132	0	147	0	132
14	0	162	0	132	0	130	0	137	0	158	0	158	0	158	0	158	0	158	0	158
15	0	162	0	140	0	134	0	143	0	158	0	160	0	160	0	158	0	160	0	158
16	0	167	0	144	0	140	0	163	0	176	0	187	0	163	0	176	0	187	0	163
17	0	169	0	148	0	141	0	170	0	190	0	198	0	170	0	190	0	198	0	170
18	0	169	0	151	0	143	0	177	0	194	0	202	0	194	0	194	0	202	0	194
19	0	175	0.5	204	0	149	2.0	192	0	199	0	281	0	199	0	199	0	281	0	199
20	0	193	0	209	0	163	0	217	0	207	0	207	0	207	0	207	0	207	0	207
21	0				0	222	0	252	0	264	0	264	0	264	0	264	0	264	0	264
22	0				0		†	265	0	265	0	265	0	265	0	265	0	265	0	265
23	0				0				0		0		0		0				0	
24	0				0				0		0		0		0				0	

Tuberculin reactions in brackets were not sharp. Tuberculin reactions of 2 mm and 5 mm shown in italics indicate that the animals have been included in groups 0 < TR < 10 and 20 ≤ TR < 5 mm respectively in Table 3. Each figure is the mean of two reactions.

* Animal with tuberculosis in test < III

† Animal not included in challenge

‡ Animal died before challenge

APPENDIX TABLE 1

Survival Time (in Days) after Challenge with Virulent Tubercle Bacilli and Size of Tuberculin Reaction (in mm) to 250 TU at Time of Challenge of Non Vaccinated Guinea Pigs and Guinea Pigs vaccinated with 10 mg BCG Vaccine at Specified Intervals before Challenge

Order of Survival	Non vaccinated controls						Vaccination period											
	Cr 1 wt 2.0 300 g			Gr II wt 500 500 g			2 weeks		4 weeks		6 weeks		8 weeks		10 weeks		12 weeks	
	Tuber- culin reac- tion	Survival time	Tuber- culin reac- tion	Tuber- culin reac- tion	Survival time	Survival time	Tuber- culin reac- tion	Survival time	Tuber- culin reac- tion	Survival time	Tuber- culin reac- tion	Survival time	Tuber- culin reac- tion	Survival time	Tuber- culin reac- tion	Survival time	Tuber- culin reac- tion	Survival time
1	0	76	0	0	35*	78	0	75	0	0.5	87	0	79	0	56	14.0	23*	
2	0	77	0	0	91	80	0	80	0	(1.5)	92	0	96	0	89	(2.0)	81	
3	0	77	0	0	92	91	0	106	0	(2.0)	96	0	102	0	97	(2.5)	86	
4	0	83	0	0	99	91	0	107	0	(2.0)	96	0	102	0	99	0	94	
5	0	88	2.0	0	102	97	1.0	110	1.5	1.5	98	0	106	0	100	0	100	
6	0	91	0	0	109	99	0	99	0	0	99	0	107	0	101	0	106	
7	0	95	0	0	113	100	0	117	0	0	104	0	113	0	101	(12.0)	109	
8	0	99	0	0	115	104	0	123	(7.5)	(2.0)	114	0	115	0	105	0	113	
9	0	117	0	0	119	104	0	124	0	(2.0)	116	3.5	132	0	107	1.5	113	
10	0	119	0	0	120	106	(3.0)	127	(1.5)	0	121	0	137	0	109	(2.5)	118	
11	0	140	0.5	0	121	110	(2.0)	130	1.0	0	127	(5.0)	142	0	111	0	130	
12	0	142	(2.5)	0	125	127	0	132	0	0	131	0	147	0	117	0	132	
13	0	151	0	0	132	130	(1.0)	137	0	(3.0)	152	0	158	0	122	0	144	
14	0	162	0	0	140	134	1.0	143	0	2.0	158	0	160	(15.5)	132	(5.5)	148	
15	0	162	0	0	144	140	0	163	0	0	176	0	187	0	143	(14.0)	154	
16	0	167	0	0	148	141	0	170	0	0	190	1.0	198	0	145	(1.0)	155	
17	0	169	0	0	151	147	0	177	0	7.0	194	0	232	0	146	0	159	
18	0	169	0.5	0	204	149	1.5	192	2.0	0	199	3.5	281	0	118	0	169	
19	0	175	0	0	209	163	0	217	0	0	207	0	-§	0	151	0	171	
20	0	193	0	0	222	222	0	252	0	(2.0)	264	0	-§	0	165	0	173	
21						†	0	265	0	0	276	0	-	0	172	0	195	
22													-	0	172	0	197	
23														13.5	191	0	211	
24																		

Tuberculin reactions in brackets were not sharp. Tuberculin reactions of 2 mm and 5 mm shown in italics indicate that the animals have been included in groups 0 < TR < 2.0 and 2.0 ≤ TR < 5 mm respectively in Table II. Each figure is the mean of two reactions.

* Animal with tuberculosis index < III

† Animal not included in challenge

§ Animal died before challenge

APPENDIX TABLE 2

Survival Time (in Days) after Challenge with Virulent Tubercle Bacilli and Size of Tuberculin Reaction (in mm) to 250 TU at Time of Challenge of Guinea Pigs Vaccinated with 10 mg BCG Vaccine at Specified Intervals before Challenge

Vaccinated at birth														
Order of survival	2 weeks		4 weeks		6 weeks		8 weeks		10 weeks		12 weeks			
	Tuber- culin reaction	Survival time	Tuber- culin reaction	Survival time	Tuber- culin reaction	Survival time	Titer- culin reaction	Survival time	Tuber- culin reaction	Survival time	Tuber- culin reaction	Survival time		
1	0	75	0	■	0	96	(15.0)	86	(15.5)	96	15.0	121		
2	0	86	1.5	101	(4.5)	101	14.5	115	8.0	98	14.5	123		
3	0	86	0	101	0	105	(8.0)	121	(10.0)	116	(14.0)	137		
4	0	92	(15.0)	102	(3.0)	105	(13.5)	122	13.0	124	12.0	138		
5	0	99	0	104	0	109	0	124	0	127	13.5	139		
6	0	100	(12.0)	106	1.0	110	(13.5)	128	0	127	16.5	148		
7	(1.5)	101	0	120	0	113	(5.0)	130	12.5	137	18.0	163		
8	0.5	104	3.0	128	11.0	121	(9.5)	132	0	141	15.5	184		
9	0	105	0	136	0	122	14.0	133	1.5	142	(17.0)	105		
10	0	105	0.5	138	0	137	(12.0)	142	13.0	145	17.0	105		
11	1.0	110	0	148	(4.5)	139	(6.0)	152	0.5	155	(21.0)	165		
12	0	117	(4.5)	148	11.0	144	15.5	154	(15.5)	159	16.0	176		
13	0	121	7.0	152	0	147	14.5	174	(15.0)	161	14.5	176		
14	0	122	(4.0)	155	14.5	153	(14.0)	182	(11.5)	164	17.0	184		
15	0	127	2.0	157	0	159	(12.0)	182	(14.0)	164	17.0	199		
16	0	139	0	160	(11.0)	161	(2.0)	183	(14.5)	168	(13.5)	216		
17	0	140	1.5	184	0	180	3.5	188	13.5	176	14.5	245		
18	2.0	149	(9.0)	185	0	184	12.5	195	0	181	(7.5)	248		
19	(1.0)	145	(9.0)	201	0	192	10.5	199	16.0	183	15.5	251		
20	3.0	160	3.0	210	0	221	0	225	(13.5)	183	14.5	252		
21	0	161	(6.0)	217	0	224	(8.5)	232	(15.0)	184	12.5	254		
22	1.0	202	(5.0)	266	(10.5)	253	0	268	15.5	186	14.5	329		
23									15.0	189	0	334		
24									14.5	209	(13.5)	362		

24 Tuberculin reactions in brackets were not sharp. Tuberculin reactions of 2 mm and 5 mm shown in *italics* indicate that the animals have been included in groups $0 < \text{TR} < 2.0$ and $2.0 \leq \text{TR} < 5$ mm respectively in Table 3. Each figure is the mean of two reactions.

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Mumps virus antigen For skin tests and cytotoxicity studies the virus suspension was inactivated in water bath at 56° C for 25 minutes

Allantoic fluid Normal allantoic fluid was treated in exactly the same way as the infected allantoic fluid

Tuberculin A batch of Old Tuberculin (OT)⁴—with strength 1.45 standard—prepared from a bovine strain K and without preservatives and one lot of purified protein derivate (PPD)⁵, likewise without preservatives was used

Lipopolysaccharides from *Serr marcescens* (S₃)⁶ Culture used for preparation of this batch was grown in a synthetic medium with glucose as carbon source⁷ The preparation was made according to the method described for Coli 9 in a previous paper (10) The protein impurities of the preparation amounted to 0.47 per cent of dry weight

Animals

27 male guinea pigs weighing 400–600 gm were sensitized according to the method described by Glasgow & Morgan (2) 0.03 ml of the concentrated infected allantoic fluid was injected into the anterior chamber of the eye and 0.1 ml was dropped into the nasal passages of the animal. Vaccination with BCG and injection of killed *M. tuberculosis* paraffin oil (10) 61 untreated male guinea pigs weighing 400–600 gm were used as controls

The method of experimentation

The skin reactivity towards heat inactivated mumps virus and tuberculin was studied in mumps virus sensitized as well as in tuberculin sensitized animals. Normal controls were included. The guinea pigs sensitized with mumps virus were tested six weeks after the sensitization intracutaneously with 0.1 ml of undiluted mumps virus antigen (2 000 HAU per ml). A few of the animals were also tested by intracutaneous injection of 0.1 ml of OT diluted 1:100. Allantoic fluid was used as control. The tuberculin sensitized animals were tested by intracutaneous injection of 0.1 ml of OT diluted 1:100 and a few of them also by injection of mumps virus antigen as described above for virus sensitized animals. The reactions were read 24 and 48 hours after the challenge.

The determination of the cytotoxicity of the various preparations studied was based on the measurements of inhibition of leukocytic migration in the presence and absence respectively of these substances according to a method used in previous experiments (7–10). The cytotoxicity was expressed in cytotoxic indices. The concentrations of the preparations used in migration tests were determined by titrating the maximum dose that did not appreciably inhibit the migration of leukocytes from normal animals (Fig. 1, Table 1 and 5).

Statistical analysis⁸

For each preparation in experiments with normal and sensitized animals and compared differences have been tested for each preparation studied between its activity on leukocytes from mumps virus sensitized and normal animals, between its activity on leukocytes from tuberculin sensitized and normal animals and finally between its activity on leukocytes from mumps virus sensitized and tuberculin sensitized animals. Each test has been carried out on the significance level of 0.1 per cent. Thus the total significance level is the

⁴ Kindly made available by Dr L. Silverstolpe, the State Bacteriological Laboratory, Stockholm

⁵ Generously supplied by Parke Davis & Co., Detroit, USA

⁶ The *Serr marcescens* strain was generously supplied by the courtesy of Dr W. J. Shear, National Institutes of Health, Bethesda, USA

⁷ The bacteria used for preparation of S₃ were grown in mass culture at the Bacteriological Dept., Karolinska Institutet, Stockholm, by the courtesy of Dr C. G. Heden

⁸ Mr Staffan Ekblom of the Statistical Research Group at the University of Stockholm has given advice on the statistical analysis

significance that any one of the observed differences has been due to chance is approximately equal to 1 per cent. This analysis made it possible to investigate the specific and nonspecific activity of the same preparation. In addition the specific and nonspecific sensitivity of leukocytes from each group of animals was compared by analysis of covariance.

TABLE 1
Cytotoxic Indices of PPD and Allantoic Fluid for Leukocytes from Normal Guinea Pigs

Animal	Date 1956	Control migration in 18 hrs	PPD		Allantoic fluid 1:10 or 1:20
			10 ~ ml	1 ~ ml	
✓ 514	4-21	170			1.03
✓ 517	4-24	136		-	1.06
✓ 567	4-27	183			0.98
✓ 604	5-29	222			1.00
✓ 666	3-1	264		0.89	-
✓ 722	3-26	201	0.90	-	-
✓ 724	3-1	172		1.03	-
✓ 725	3-3	192	1.03		-
✓ 725	2-26	206	0.58		-
✓ 725	3-1	178		0.90	-
✓ 726	2-26	201	0.80		-
✓ 729	5-2	210	0.87	0.99	-
✓ 730	2-26	150	1.00		-
✓ 731	3-2	170	0.91	1.03	-
✓ 732	3-2	209	0.83	1.00	-
✓ 733	3-2	177	0.99	1.01	-
✓ 735	3-4	219	0.93		-
✓ 736	3-4	207	0.90	-	-
✓ 737	3-7	227	0.78		-
✓ 739	3-21	192	1.06		-
✓ 741	3-21	225	0.68		-
✓ 743	3-11	163	0.73	-	-
✓ 744	3-11	167	0.95		-

RESULTS

22 animals inoculated with mumps virus were tested with mumps virus antigen and all of them were found to have developed a state of delayed hypersensitivity as determined by their skin reactivity. The mean diameter of the area of skin induration 48 hours after the challenge was 11 mm. In several of the reaction sites a central zone of necrosis developed (Table 2). Six of the mumps sensitized animals were tested with OT. None of them showed a positive skin reaction to this antigen.

All 45 tuberculin sensitized guinea pigs reacted to the intracutaneous injection of OT. The mean diameter of skin induration was 30 mm. Nearly all animals developed central zones of necrosis and hemorrhage in the reaction sites. Five of these tuberculin sensitized animals were tested with mumps virus antigen and reacted negatively to this preparation. None of the control animals developed positive skin reactions to either preparation.

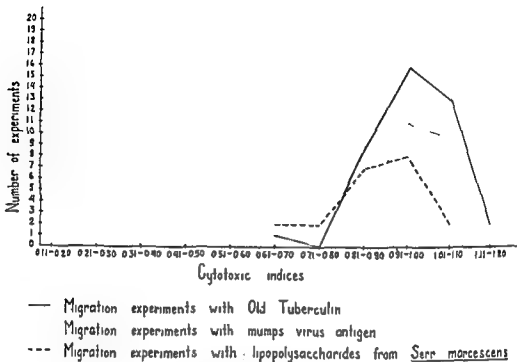


Fig 1

Frequency Distribution of Cytotoxic Indices for Old Tuberculin Mumps Virus Antigen and *Serr. marcescens* Lipopolysaccharides in Experiments with Normal Guinea Pigs

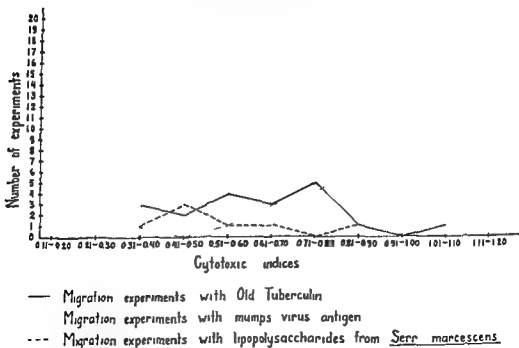


Fig 2

Frequency Distribution of Cytotoxic Indices for Old Tuberculin Mumps Virus Antigen and *Serr. marcescens* Lipopolysaccharides in Experiments with Tuberculin Sensitized Guinea Pigs

TABLE 2

Delayed Type Skin Reactions in Mumps Virus Sensitized Guinea Pigs Challenged with Mumps Virus Antigen

Animal	24 hours		48 hours	
	Infiltration	Necrosis	Infiltration	Necrosis
	mm	mm	mm	mm
MS 559	8 × 9	—	7 × 8	—
MS 560	10 × 11	—	7 × 8	2 × 3
MS 561	10 × 12	—	12 × 15	—
MS 562	12 × 12	—	9 × 11	—
MS 564	6 × 7	—	5 × 6	—
MS 565	12 × 15	—	14 × 15	2 × 4
MS 577	15 × 15	—	9 × 10	—
MS 578	12 × 12	—	11 × 11	—
MS 579	11 × 11	—	10 × 17	—
MS 580	12 × 15	2 × 3	5 × 5	—
MS 581	10 × 12	1 × 2	10 × 10	—
MS 582	10 × 12	—	5 × 5	—
MS 583	14 × 14	2 × 3	7 × 10	—
MS 584	20 × 20	—	9 × 17	—
MS 585	12 × 12	2 × 3	5 × 8	2 × 2
MS 586	9 × 9	—	5 × 5	—
MS 697	12 × 14	2 × 4	10 × 11	1 × 2
MS 698	12 × 12	2 × 4	10 × 11	1 × 2
MS 699	7 × 12	—	6 × 7	—
MS 700	7 × 8	2 × 3	7 × 9	—
MS 703	10 × 12	1 × 2	10 × 12	1 × 7
MS 704	7 × 14	—	8 × 10	—
Mean diameter	11.5		9.0	

Leukocyte Migration Inhibition

The cytotoxic indices are presented in frequency tables, with the exception of indices for PPD and allantoin fluid which were too few to be accounted for in this way.

The migration of leukocytes deriving from tuberculin sensitized animals was inhibited by the products of tubercle bacteria examined in the present experiments (OT and PPD), as demonstrated by low cytotoxic indices for these preparations (Fig 2, Table 3). The leukocytes from tuberculin sensitized guinea pigs were sensitive not only to products of tubercle bacteria but also to products of other bacteria. The differences were of significance.

Similarly the migration of leukocytes from mumps sensitized guinea pigs was inhibited not only by specific mumps virus antigen but also by OT and lipopolysaccharides from *Serratia marcescens* (Fig 3). It may be seen from Table 5, that the mean cytotoxic indices of these three substances for mumps sensitized animals were considerably lower than the difference was

TABLE 3

Cytotoxic Indices of PPD for Leukocytes from Tuberculin Sensitized Guinea Pigs

Animal	Date 1949	Control migration mm 18 hrs	PPD	
			10 γ ml	1 γ ml
TS 705	3-24	2.09	-	0.89
TS 706	3-24	1.74	-	0.78
TS 714	3-10	2.22	0.49	-
TS 715	3-1	2.88	-	0.73
TS 716	3-1	2.31	-	0.62
TS 729	3-1	3.28	-	0.85
TS 730	3-1	1.20	-	0.51
TS 732	3-2	3.15	0.56	0.65
TS 736	3-2	2.73	0.67	0.77
TS 737	3-2	3.11	0.61	0.76
TS 739	3-2	3.32	0.37	0.72
TS 740	3-11	1.86	0.77	-
TS 741	3-7	2.20	0.50	-
TS 742	3-7	3.80	0.54	-
TS 746	3-7	4.43	0.51	-
TS 751	3-9	3.69	0.50	-

TABLE 4

Cytotoxic Indices of PPD and Allantoic Fluid for Leukocytes from Mumps Virus Sensitized Guinea Pigs

Animal	Date 1949	Control migration mm 18 hrs	PPD		Allantoic fluid 1:10 or 1:20
			10 γ ml	1 γ ml	
MS 577	5-27	1.82	-	-	1.02
MS 578	5-27	2.30	-	-	0.92
MS 579	5-27	1.99	-	-	0.99
MS 581	5-28	2.81	-	-	0.58
MS 582	5-28	2.81	-	-	0.98
MS 583	5-29	2.29	-	-	0.88
MS 584	5-29	2.91	-	-	1.04
MS 585	5-29	1.84	-	-	1.01
MS 586	5-29	2.63	-	-	0.79
MS 697	3-3	2.25	0.72	-	-
MS	3-14	1.86	-	0.84	-
MS	5-5	2.75	-	-	1.09
MS 698	3-14	1.49	0.62	0.99	-
MS	5-5	2.28	-	-	1.29
MS 699	3-3	2.95	0.63	-	-
MS	3-14	2.07	-	0.87	-
MS 700	3-14	2.04	0.71	0.99	-
MS	5-5	1.86	-	-	1.13
MS 702	3-4	2.37	0.77	-	-
MS	3-14	1.80	-	0.70	-
MS 703	6-10	1.50	0.61	-	-
MS 704	3-14	2.05	1.08	1.15	-
MS	5-9	2.76	-	-	0.95

TABLE 5

Probability Values for the Cytotoxic Effect of Homologous and Heterologous Bacterial and Viral Products on the Migration of Leukocytes from Mumps-Virus-Sensitized Tuberculin Sensitized and Normal Guinea Pigs

Experiment	Concentration	N ₁	N ₂	N ₃	N ₄	N ₅	t _(1, 2)	t _(1, 3)	t _(1, 4)	t _(1, 5)
Mumps virus antigen	100 HAU/ml	25	19	18	0.97	0.50	0.77	15.48	<0.001	5.83
Old tuberculin	1:200	9	7	0.84	0.60	0.62	3.86	<0.01	<0.001	<0.001
	1:1000	41	26	19	0.96	0.73	7.00	9.38	<0.001	2.11
PPD	10 γ/ml	16	7	10	0.87	0.73	2.18	6.39	<0.001	2.72
	1 γ/ml	7	6	10	0.98	0.92	0.95	5.39	<0.001	2.85
S ₁	1:25 γ/ml	21	15	7	0.89	0.67	3.92	6.28	<0.001	1.59
Atlantio fluid	1:10 or 1:20	4	13	—	1.05	0.97	0.91	—	—	—

N₁ = number of experiments with leukocytes from normal animals

N₂ = number of experiments with leukocytes from mumps virus sensitized animals

N₃ = number of experiments with leukocytes from tuberculin sensitized animals

N₄ = mean cytotoxic index for experiments with leukocytes from normal animals

N₅ = mean cytotoxic index for experiments with leukocytes from mumps virus sensitized animals,

N₅ = mean cytotoxic index for experiments with leukocytes from tuberculin-sensitized animals

t = probability values calculated according to Student's t-test

$$t = \frac{(\bar{N}_1 - \bar{N}_2)}{\sqrt{\frac{(N_1 + N_2 - 2)(N_1 \cdot N_2)}{(N_1 + N_2)(\bar{N}_1 - \bar{N}_2)^2 + \sum (N_i - \bar{N}_i)^2}}}$$

t_(1, 2) = probability values for differences between the mean cytotoxic indices for normal and mumps sensitized leukocytes

t_(1, 3) = probability values for differences between the mean cytotoxic indices for normal and tuberculin sensitized leukocytes

t_(1, 5) = probability values for differences between the mean cytotoxic indices for mumps sensitized and tuberculin-sensitized

leukocytes

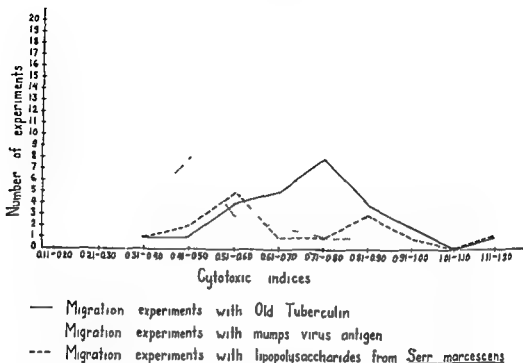


Fig. 3

Frequency Distribution of Cytotoxic Indices for Old Tuberculin, Mumps Virus Antigen and *Serr. marcescens* Lipopolysaccharides in Experiments with Mumps Virus-Sensitized Guinea Pigs

A comparison of the specific and nonspecific activity of the preparations studied showed that the cytotoxic effect of mumps virus antigen on leukocytes from mumps-sensitized animals was significantly more pronounced than on leukocytes from tuberculin-sensitized animals. A corresponding tendency for the specific and nonspecific activity of OT was observed (Table 5).

When comparing the sensitivity of leukocytes to homologous and heterologous preparations, respectively, regression lines were written according to the formula $y_1 = a_1 + b_1(x_1 - x_1)$ and $y_2 = a_2 + b_2(x_2 - x_2)$ where the independent variable x was the inherent rate of migration⁹ and the dependent variable y was the cytotoxic index. Thus, two lines were written for each group of animals, one each for the homologous and the heterologous preparations. The coefficients of regression were calculated according to the formula

$$b_1 = \frac{\sum x_1 y_1 - \frac{(\sum x_1)(\sum y_1)}{n_1}}{\sum x_1^2 - \frac{(\sum x_1)^2}{n_1}}$$

⁹ The distance of leukocyte migration in control capillary tubes after 18 hours was termed the inherent rate of migration. The inherent rates of migration of leukocytes from normal and sensitized animals are given in Fig. 4.

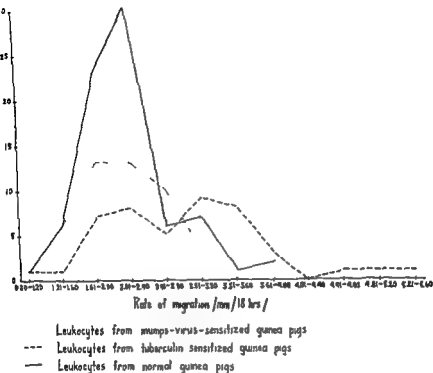


Fig 4
Frequency Distribution of Inherent Rates of Leukocyte Migration

and in experiments with mumps-sensitized animals were found to be -0.0056 for mumps virus antigen and -0.0876 for OT. In experiments with tuberculin-sensitized animals, the coefficients of regression were -0.0259 for OT and -0.0194 for mumps virus antigen. The significance of these four values was tested by Student's *t*-test according to the formula

$$t = \frac{b_1}{\epsilon b_1}$$

ϵb_1 being the standard error, which was obtained by

$$\epsilon b_1 = \frac{S_1}{\sqrt{\sum x^2 - \frac{(\sum x)^2}{n}}}$$

the standard deviation *S* around the respective regression line being

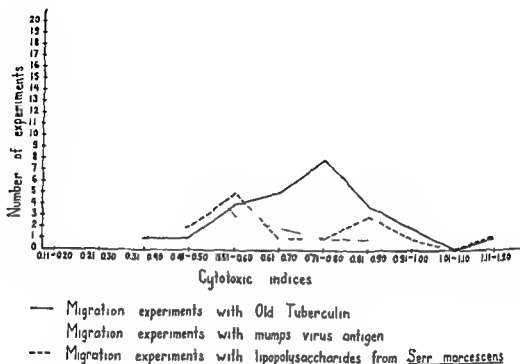


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¹ The distance of leukocyte migration in control capillary tubes after 18 hours was termed the inherent rate of migration. The inherent rates of migration of leukocytes from normal and sensitized animals are given in Fig. 4.

$$t = \frac{d}{sd} \quad (n_1 + n_2 - 4 \text{ degrees of freedom})$$

The standard error sd of d was calculated as follows

$$sd = S_{t+2} \sqrt{\frac{1}{n_1} + \frac{1}{n_2} + \frac{(\bar{x}_1 - \bar{x}_2)^2}{\sum x_1^2 - \frac{(\sum x_1)^2}{n_1} + \sum x_2^2 - \frac{(\sum x_2)^2}{n_2}}}$$

t was found to be 4.56 for mumps sensitized animals, degrees of freedom 41 and 2.56 for tuberculin-sensitized animals, degrees of freedom 33. The two regression lines for mumps virus sensitized animals are thus not identical and the vertical distance between them, 1.2, the difference between the activity of the homologous and the heterologous preparation respectively, highly significant. For tuberculin-sensitized animals this difference is probable.

DISCUSSION

Investigation with tuberculin-sensitized as well as with mumps-virus sensitized animals tallies with this opinion.

The results of migration studies present, on the other hand, a different and more complex picture.

The fact that the cells from tuberculin sensitized animals were found to be sensitive not only to homologous products of tubercle bacteria and heterologous lipopolysaccharides of *Serr. marcescens*, in confirmation of previous results from this laboratory (10), but also to mumps virus, broadens the common denominator of substances capable of eliciting nonspecific hypersensitivity *in vitro*. The results obtained in the study of leukocytes from mumps sensitized animals demonstrated a non-specificity to heterologous products similar to that encountered in tuberculosis. Out of bacterial products tested, OT was definitely cytotoxic for these leukocytes. The cytotoxicity of PPD for leukocytes from mumps sensitized animals was not statistically significant. The number of experiments with PPD was however rather limited. Lipopolysaccharides from *Serr. marcescens* had a clear cytotoxic effect on mumps-sensitized leukocytes, semblable to its effect on tuberculin-sensitive cells. Thus an increased reactivity of cells towards this heterologous lipopolysaccharide is a phenomenon not restricted to the immunological state present in tuberculosis but demonstrable also in another state of delayed hypersensitivity.

It is difficult to present a complete explanation of the above mentioned results on the basis of the data obtained in this study. Certain

$$S_1 = \sqrt{\frac{1}{n-2} \left\{ \sum y_1^2 - \frac{(\sum y_1)^2}{n_1} - \frac{[\sum x_1 y_1 - \frac{(\sum x_1 \sum y_1)}{n_1}]^2}{\sum x_1^2 - \frac{(\sum x_1)^2}{n_1}} \right\}}$$

None of the coefficients of regression was found to be significant indicating that in the present series of experiments the cytotoxic indices were not likely to have been influenced by the rate of migration. The next step was to compare the two regression lines for each group of animals by analysis of covariance in order to see whether the vertical distance between the two lines was constant i.e. without relation to the dependent variable of the lines. A hypothesis $b_1 = b$ was tested by Student's t test as follows

$$t = \frac{b_1 - b_2}{sb_1 - b_2} \quad (n_1 + n_2 - 4 \text{ degrees of freedom})$$

where b represents the standard error for b_1 and equals

$$sb_1 - b_2 = \frac{S_{1+2}}{\sqrt{\sum x_1^2 - \frac{(\sum x_1)^2}{n_1} + \sum x_2^2 - \frac{(\sum x_2)^2}{n_2}}}$$

where

$$S_{1+2} = \sqrt{\frac{(n_1-1) S_1^2 + (n_2-1) S_2^2}{n_1 + n_2 - 4}}$$

The hypothesis was accepted t being not significant for both mumps sensitized ($t = 0.67$ degrees of freedom 41) and tuberculin sensitized animals ($t = 0.10$ degrees of freedom 33). As the two regression lines for both groups of animals were unlikely to cross each other the comparison was then continued in respect to the vertical distance between the lines in order to see whether this distance was equal to 0 or not. This distance can be written

$$d = y_1 - y_2 - b(x_1 - x_2)$$

where the common slope of the two lines is

$$b = \frac{\sum x_1 y_1 - \frac{\sum x_1^2 \sum y_1}{n} + \sum x_2 y_2 - \frac{\sum x_2^2 \sum y_2}{n}}{\sum x_1^2 - \frac{(\sum x_1)^2}{n} + \sum x_2^2 - \frac{(\sum x_2)^2}{n}}$$

The hypothesis $d = 0$ was tested by Student's t test according to the formula

$$t = \frac{d}{\text{ed}} \quad (n_1 + n_2 - 4 \text{ degrees of freedom})$$

The standard error ed of d was calculated as follows

$$\text{ed} = S_{1+2} \left\{ \frac{1}{n_1} + \frac{1}{n_2} + \frac{(x_1 - x_2)^2}{\sum x_1^2 - \frac{(\sum x_1)^2}{n_1} + \sum x_2^2 - \frac{(\sum x_2)^2}{n_2}} \right\}$$

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It is difficult to present a complete explanation of the above mentioned results on the basis of the data obtained in this study. Certain

aspects of the observed specific and nonspecific hypersensitivity *in vitro* should nevertheless be discussed

The quantitative data obtained in this investigation do not support the assumption that the displayed hypersensitivity might solely be due to an increased vulnerability of the cells towards a variety of noxious agents and thus completely nonspecific in an immunological sense. An overall nonspecifically increased vulnerability to diverse agents should thus imply that the relative inhibitory activity of these agents would essentially depend on their inherent cytotoxicity and not on the kind of sensitization (mumps, tuberculin, etc.) to which the animals have been subjected. The present study demonstrates, however, that while the cytotoxicity of the mumps virus antigen used, was more pronounced for leukocytes from mumps-sensitized animals, the cytotoxicity of OT on the other hand proved stronger when tested on leukocytes from the tuberculin-sensitized animals. Taken another way, the results showed that leukocytes deriving from mumps-sensitized animals were more sensitive to the mumps virus antigen than to OT, whereas leukocytes from tuberculin-sensitized animals reacted more strongly to OT than to the mumps virus antigen. This latter comparison between the cytotoxicity of homologous and heterologous substances, respectively, on leukocytes from both groups of animals demonstrates also that the cytotoxicity of the two preparations and the degree of the sensitization, as measured by migration tests, were of a comparable order of magnitude. On the basis of the above-mentioned findings, it is reasonable to assume that the cytotoxicity caused by homologous products, or at least a part of it, is immunologically specific.

Darlington & Sherago (1) and Heilman *et al.* (3) studied the delayed type hypersensitivity in experimental tuberculosis as well as in brucellosis using protein fractions of the sensitizing bacteria. They found the cytotoxic effect of the two preparations to be specific and without cross reactions. Although the experiments were not performed with pure protein preparations it is possible that the specific cytotoxicity demonstrated by the above-mentioned workers, as well as the homologous cytotoxicity shown in this study, is protein-linked. Such a specific hypersensitivity *in vitro* to protein components might be a phenomenon analogous to the tuberculin sensitivity *in vivo*, which is generally considered to be due to specific tuberculo-protein.

The overlapping reactions of agents of different origin, demonstrated in this report, could be due to partial antigens common to all of them, reacting with sessile or humoral antibodies. The presence of factors common to tubercle bacteria, *Serratia marcescens* as well as mumps virus is possible but not very probable.

Another possibility would be that the *in vitro* hypersensitivity to heterologous agents is a phenomenon based on mechanisms different from those controlling the specific hypersensitivity to homologous agents. A completely nonspecific vulnerability was considered as less

likely to be the common mechanism of sensitivity to both homologous and heterologous substances. Such an increased nonspecific reactivity might however be the special basis of hypersensitivity reactions to heterologous preparations. There is some evidence bearing on this point. It has been reported that cells from BCG vaccinated animals compared to cells from nonvaccinated animals (8) show an increased resistance towards mechanical trauma. An investigation performed in this laboratory has confirmed these findings (6). An altered reactivity of this type is unlikely to be immunological in nature. It might be the manifestation of some general change in the reactivity of cells deriving from hosts displaying delayed type hypersensitivity. This change may also include a nonspecifically increased reactivity to certain biological constituents.

Regarding the nature of the principle or principles responsible for the nonspecifically altered reactivity *in vitro* direct information is lacking at present time. It should be mentioned however that the lipopolysaccharide preparation from *Serratia marcescens* which was a substance heterologous with respect to the sensitization of both groups of animals had a very low content of protein in the concentration used for migration studies (amounting to 0.006 mg per ml). This suggests that the lipopolysaccharide bulk of this preparation rather than its protein impurity might have been responsible for its nonspecific cytotoxic effect. Other studies to be published separately (11) showing that a high molecular dextran also affected leukocytes from tuberculin sensitized animals nonspecifically tallies with this view. In other words there seems to be some evidence of hypersensitivity reactions *in vitro* of cells deriving from animals displaying delayed type hypersensitivity being elicited by substances which are nonprotein in nature.

SUMMARY

Leukocytes from guinea pigs sensitized against mumps virus antigen and tuberculin were tested *in vitro* for their sensitivity to homologous and heterologous preparations. The migration of leukocytes from tuberculin sensitized animals was inhibited by PPD and OT.

Mumps virus antigen and by OT and lipopolysaccharides from *Serratia marcescens*. For both groups of animals the sensitivity to the homologous substance was more pronounced than to the heterologous one. It is suggested that the *in vitro* sensitivity of cells from animals displaying delayed type hypersensitivity or at least a part of it to products deriving from the sensitizing agent might be specific in an immunological sense whereas the sensitivity towards heterologous products might be a result of a nonspecifically altered reactivity of these cells.

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THE INHIBITORY EFFECT OF NATIVE DEXTRAN ON THE IN VITRO MIGRATION OF LEUKOCYTES FROM TUBERCULIN-SENSITIZED GUINEA PIGS¹

By

J. WASSERMAN and TH. PACKALÉN

Received 21 VIII 61

Earlier studies of cytotoxicity phenomena in states of delayed type hypersensitivity have shown not only the specific hypersensitivity to products deriving from the sensitizing agent but also the presence of an increased reactivity *in vitro* to heterologous biological products (8, 9, 17, 18, 19). Among preparations studied and found capable of eliciting such phenomena were lipopolysaccharide endotoxins from gram negative bacteria. The chemical constitution and biological activities of these substances have been studied rather extensively. However, the question as to which one of

is responsible for their biologic

(13, 20, 21). Ribi and coworkers

potency of different endotoxin preparations were not correlated to variations in their lipid content, and therefore they suggested that the

are known to produce effects similar to those of endotoxins, when injected into experimental animals. They cause leukopenia and fever and can be used as provocative agents in both the local and the generalized Shwartzman phenomenon (2, 14, 16). In view of these facts, it was thought to be of interest to investigate whether one of these macromolecular polysaccharides from gram positive bacteria can elicit nonspecific cytotoxicity reactions similar to those caused by endotoxins. The choice fell on native dextran.

The present paper is concerned with the results of such an investigation. The method of determining the *in vitro* cytotoxicity was the same as in previous studies (19).

¹ This investigation was supported by grants from the Swedish National Association against Heart and Chest Diseases.

MATERIALS AND METHODS

Leukocyte donors Male guinea pigs weighing 400-600 gm were sensitized by vaccination with BCG and injection of killed bovine tubercle bacteria emulsified in paraffin oil (19). The tuberculin sensitivity of the animals was controlled by intracutaneous injections of Old Tuberculin (19). Untreated male guinea pigs weighing 400-600 gm were used as controls.

Preparation A batch of native dextran of very high average molecular weight was generously supplied by Dr Björn Ingelman Pharmacia Lab Uppsala. The batch was found to contain 0.063 per cent total nitrogen—by Kjeldahl method 0.28 per cent protein—by Folin Wu method (6) 0.026 per cent total phosphor—according to the method described by R. J. L. Allen (1). The content of chloroform soluble ether insoluble lipids which amounted to 0.125 per cent was estimated by subtracting the amount of ether soluble lipids from the amount of lipids extracted from the preparation by chloroform. The method used for these determinations was the following. The dextran was hydrolyzed for 30 minutes at 100° C with 1 N HCl. This is the procedure used by Westphal & Luderitz for splitting the lipoid A from lipopolysaccharides of gram negative bacteria (22). The hydrolysate was shaken three times with chloroform and the chloroform of the extract was evaporated with a stream of carbon dioxide at room temperature. The residue was dried *in vacuo* and then treated with chloroform. The extract was filtered and the chloroform was evaporated as described above. The residue was dried, weighed and then treated with ethyl ether. The ether extract was filtered and the ether evaporated. The residue obtained was dried and weighed.

The preparation was tested in rabbits for its preparatory and provocative activity in the Shwartzman phenomenon. It was found to be active as a preparatory agent in an amount of 0.2-1 mg and as a provocative agent in an amount of 200 mg.

Method of experimentation The cytotoxicity of dextran was determined by the inhibition of leukocytic migration in presence of this substance. The cytotoxicity was expressed in cytotoxic indices in accordance with our previous investigations. The cytotoxic index for every experiment being a ratio between the mean migration value obtained with and without the preparation added to the heparinized blood from one animal. This latter value was termed control migration (19). The mean migration value for every experiment was based on determination of leukocytic migration in ten capillary tubes. The standard error for this mean migration value was calculated according to a formula

$$s = \frac{S}{\sqrt{n}}$$

where S stands for standard deviation between ten determinations of leukocytic migration in ten representative experiments with leukocytes from normal animals without dextran being added to the blood. S was calculated as follows

$$S = \sqrt{\frac{\sum s^2}{10}}$$

S_1 being the standard deviation between ten determinations of leukocytic migration in a single experiment

$$S_1 = \sqrt{\frac{\sum x^2 - \frac{(\sum x)^2}{n}}{n-1}}$$

The standard error was found to be ± 0.13 mm the average of the ten mean migration values being 3.75 mm.

■ Dr C. Weibull from the biochemical department of this laboratory has kindly performed the chemical analysis of the dextran preparation used.

TABLE 1

Cytotoxic Indices of Native Dextran for Migration of Leucocytes from Normal and Tuberculin Sensitized Guinea Pigs

Animal	Date (1960)	Control migration mm 18 hrs	Dextran mg/ml		
			100	10	1
Normal Animals					
805	8-2	3.36	0.61	0.93	0.81
806	8-2	1.44	0.41	1.01	0.87
807	8-2	1.80	0.73	0.83	0.90
775	8-5	2.58	0.76	0.91	0.80
776	8-5	1.86	0.83	0.94	0.96
808	8-5	3.03	0.80	0.60	0.63
809	8-5	2.78	0.73	1.04	0.96
810	8-5	2.92	0.71	0.84	0.73
811	8-9	2.49	0.83	1.03	0.97
813	8-9	2.88	0.72	0.81	0.82
814	8-9	2.20	0.79	1.02	1.09
815	8-9	2.24	0.72	0.82	0.86
816	8-9	1.67	0.90	0.99	0.99
774	9-12	1.99	0.66	0.74	0.83
804	9-14	2.02	0.73	0.79	0.94
802	9-19	2.33	0.45	0.66	0.75
812	9-26	2.68	0.67	0.84	1.07
829	9-28	3.58	0.65	0.74	0.94
830	9-28	2.11	0.59	0.74	0.89
831	9-30	2.24	0.63	0.90	0.92
765	10-3	5.20	0.45	0.61	0.76
776	10-3	5.38	0.61	0.75	0.88
833	10-5	3.33	0.68	0.73	0.98
835	10-5	3.91	0.49	0.75	0.80
837	10-9	2.94	0.67	0.79	0.77
685	10-10	2.66	0.52	0.67	0.86
686	10-10	2.80	0.49	0.68	0.78
767	10-11	5.61	0.53	0.57	0.70
Mean		2.86	0.66	0.81	0.87
Sensitized Animals					
759	8-25	2.05	0.49	0.69	0.72
764	8-25	2.18	0.46	0.61	0.64
757	8-31	1.75	0.66	0.90	0.88
755	9-12	1.99	0.63	0.66	0.88
763	9-19	3.42	0.64	0.74	0.79
762	9-25	2.03	0.51	0.56	0.65
783	9-26	2.57	0.69	0.75	0.97
788	9-26	3.00	0.70	0.76	0.89
752	9-28	5.74	0.50	0.65	0.81
754	9-28	2.96	0.49	0.73	0.98
753	10-3	2.89	0.51	0.67	1.05
754	10-3	4.69	0.32	0.55	0.65
765	10-3	5.92	0.41	0.64	0.76
766	10-3	5.37	0.37	0.50	0.51
768	10-5	5.96	0.58	0.70	0.93
769	10-5	2.64	0.49	0.66	0.69
771	10-9	4.47	0.49	0.66	0.77
773	10-9	4.13	0.43	0.70	0.80
775	10-10	4.29	0.46	0.56	0.53
777	10-10	2.54	0.54	0.71	0.97
Mean		3.53	0.52	0.67	0.79

RESULTS

It may be seen from Table 1 that the migration of leukocytes deriving from tuberculin-sensitized animals in the presence of dextran was inhibited as compared to the migration of leukocytes from normal animals. This inhibition is expressed by lower cytotoxic indices for leukocytes from tuberculin-sensitized animals. The differences between mean cytotoxic indices for the two series of animals are highly significant when the concentration of 100 and 10 mcg per ml of dextran was used and probable when 1 mcg per ml of dextran was used (Table 2).

TABLE 2

Probability Values for the Cytotoxic Effect of Native Dextran on the Migration of Leukocytes

Amount of dextran mcg ml	N_1	N_2	\bar{X}_1	\bar{X}_2	t	P
1	28	20	0.87	0.79	2.12	<0.05
10	28	20	0.81	0.67	3.98	<0.001
100	28	20	0.66	0.52	3.98	<0.001

N_1 —number of experiments with leukocytes from normal animals

N_2 —number of experiments with leukocytes from tuberculin sensitized animals

\bar{X}_1 —mean cytotoxic index for experiments with leukocytes from normal animals

\bar{X}_2 —mean cytotoxic index for experiments with leukocytes from tuberculin sensitized animals

Probability values calculated according to Student's t test

$$t = (\bar{X}_1 - \bar{X}_2) \sqrt{\frac{(N_1 + N_2 - 2) N_1 N_2}{N_1 + N_2 [S(\bar{X}_1 - \bar{X}_1)^2 + S(\bar{X}_2 - \bar{X}_2)^2]}}$$

As it was found at the same time that the inherent rate of migration³ of leukocytes from tuberculin-sensitized animals was faster than that of leukocytes from normal animals (Table 1, Fig. 1), a question arose as to what extent the inherent rate of migration affects the degree of inhibition. This tendency towards longer migration on the part of leukocytes deriving from tuberculin-sensitized guinea pigs, as compared with those from normal ones, was observed to some extent also in previous experiments but was never so pronounced as in the present experimental series.

If the degree of the inhibition of migration is influenced by the inherent rate of migration, then it is of primary importance to know whether the differences between the cytotoxic indices for respective groups of animals are due solely to the differences in the inherent rate of migration or are also caused by other factors. In order to answer

³ That means the distance of leukocyte migration in the control capillary tubes after 18 hours, also called control migration

the first question, i.e., the relation between the inherent rate of migration and the degree of inhibition, a regression line for each group of the animals was written⁴, where the independent variable was the inherent rate of migration and the dependent variable the cytotoxic index. The experiments performed with 10 mcg per ml of dextran were chosen for this statistical analysis. The coefficients of regression b_1 and b_2 were

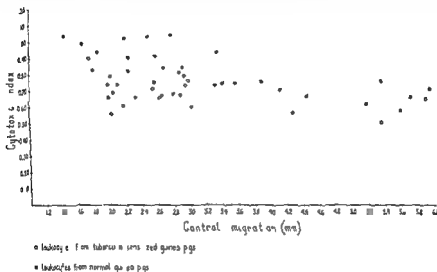


Fig. 1

Relationship between the degree of inhibition of leukocyte migration by dextran (10 mcg per ml) and the control migration

calculated and found to be $b_1 = -0.071$ for normal animals and $b_2 = -0.0245$ for tuberculin-sensitized animals. The significance of these two values was tested by Student's *t*-test. The coefficient of regression for the normal animals was significant $t = 3.38$, degrees of freedom 26. For that reason it was concluded that for leukocytes with faster migration the degree of inhibition was greater.

The next step was to compare the two regression lines by analysis of variance.

The hypothesis $b_1 = b_2$ was tested by Student's *t*-test on 1

degrees of freedom. The hypothesis $d = 0$ was tested by Student's *t*-test according to the formula

⁴ All formulae used in the following statistical analysis are given in the preceding paper (18).

$$t = \frac{d}{e_d} (n_1 + n_2 - 4 \text{ degrees of freedom})$$

It was found to be 3.52, *degrees of freedom* 44, i.e., significant, and the hypothesis could not be accepted. The two regression lines thus are not identical and the vertical distance between them, i.e., the difference between the degree of inhibition for the respective groups of animals, can be considered as significant and not related to the inherent rate of migration.

DISCUSSION

As native dextran is rather a crude preparation a critical appraisal of its degree of purity should precede any attempt to interpret the results obtained in the course of this study. Although several biological activities of native dextrans have been investigated by others, no details have hitherto been given on the presence, or the absence of impurities in preparations tested (2, 16). The data obtained by chemical analysis in this laboratory show that the preparation used in present experiments contained small quantities of substances other than dextran.

The protein content of the dextran preparation used was 0.28 per cent. This corresponds to an amount of protein in the migration experiments of 0.03 mcg per ml, which is a quantity far below those reported to have any cytotoxic activity *in vitro* (3, 4, 5, 7, 11, 15).

To our knowledge gram positive bacteria have not been reported to contain lipopolysaccharides of endotoxin nature like those occurring in gram negative bacteria. However, as the chemical analysis revealed the presence of lipids in our preparation, we could not *a priori* exclude the occurrence of lipopolysaccharides in it, either deriving from the *Leucostoc* itself or being due to a contamination. The possibility that such lipopolysaccharides have been present in the preparation used, makes it rather important to know the order of magnitude of this impurity.

In a review of chemical data on different lipopolysaccharide preparations Westphal & Luderitz state that the lipoid A fraction constitutes, on an average, about 20 per cent of the preparations. The mean phosphorus content was reported to be about 2 per cent (22). If all the lipid substances soluble in chloroform and insoluble in ether found by us were identical with lipoid A of lipopolysaccharides, then their total amount would be about 0.6 per cent. On the other hand, if all the phosphorus demonstrated to be present in the preparation was included in the lipopolysaccharides, then they would constitute about 1.3 per cent of the dry weight of the preparation. If the preparation contained any lipopolysaccharides of the same character as those of gram negative bacteria, their amount could not reasonably exceed 1 per cent. The concentration of lipopolysaccharides in the actual experiments would then be at most about 0.1 mcg per ml. This is about one tenth of the

smallest quantity of lipopolysaccharides of gram negative bacteria hitherto found in our studies to possess a nonspecific cytotoxic activity *in vitro*. On the basis of the above discussion it can be concluded that, even if the preparation contains small amounts of lipopolysaccharides, it is not likely that they could be responsible for the cytotoxic effect demonstrated in this investigation. This makes it all the more probable that the high molecular dextran *per se* is capable of eliciting hypersensitivity reactions *in vitro*, similar to those shown in our earlier studies. The fact that dextran is chemically a relatively well defined substance, in contrast to several other preparations used previously in this type of experiment, e.g., Old Tuberculin and mumps virus antigen, which contain proteins, lipids as well as polysaccharides, lends special interest to this observation. The finding is also in line with the view of Ribi and his coworkers on the relation between the chemical constitution of endotoxins and their biological activity (13). From this it should not be concluded, however, that macromolecular polysaccharides are the sole bacterial constituents capable of producing non-specific cytotoxic effects on cells from animals with delayed type hypersensitivity.

No adequate answer can be given as to why the differences in the inherent rate of migration between the tuberculin sensitized and normal leukocytes were more pronounced in the present experimental series than in our earlier experiments. The variations in leukocyte migration in present and previous experimental series could not be correlated with total or differential white blood cell counts of the final cell suspension used for capillary studies. It may be mentioned in this connection that an investigation performed at this laboratory (10) has shown that the trauma, which prolonged centrifugation involves, impairs the migration of leukocytes deriving from normal guinea pigs to a higher degree than that of leukocytes from tuberculin sensitized guinea pigs. These observations are in line with a report of Patnode and Hudgins on the increased resistance of monocytes from BCG vaccinated guinea pigs towards disruption by sonic vibration (12). However, since the method of experimentation was the same in this series as before, the degree of mechanical trauma to which the leukocytes were subject should have been of the same order of magnitude as previously. Some other factor or factors beyond our control, for example seasonal or dietary variations, may have influenced more or less the fragility of leukocytes from normal and sensitized animals respectively. Since the statistical analysis has demonstrated that the difference between the degree of inhibition of the leukocyte migration for the tuberculin sensitized and normal animals respectively, was not due solely to the variations in their inherent rates of migration, this latter phenomenon was not subject to any additional investigation in the course of the present study.

The degree of inhibition was influenced by the inherent rate of leukocyte migration. A statistical analysis of this relationship showed that the inhibitory effect of dextran was not solely due to the differences in the inherent rates of migration between the respective groups of animals.

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ANTIGEN MOSAIC OF BLOOD SERUM AND MILK IMMUNE GLOBULINS

By

LARS Å HANSON

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The presence of material in milk which is identical with or related to the blood serum immune globulins, γ -, β_2A - and β_2M - globulins, have been reported by several authors (2, 5, 6, 7, 12, 13, 14, 15, 16, 17, 18, 19, 20). Recent findings indicate a close relationship between the immune globulins of milk and of blood serum, but also definite differences are found (8, 9, 10). This report will present some comparative analyses of the milk and serum immune globulins illustrating the complicated pattern of antigenic determinants of these proteins.

MATERIAL AND METHODS

Colostrum taken within one day after parturition was used. Fresh blood plasma and several preparations of γ globulin* were employed. Preparations of Bence Jones proteins belonging to groups I and II of *Burtin et al.* (1) were kindly offered by Dr P. Burtin, Paris. (The preparations were called BJ I and BJ II).

A large number of anti colostrum, anti blood plasma and anti γ globulin immune sera were used in the investigation. Some of the anti γ globulin sera also contained antibodies against β_2A and β_2M globulins and were called anti immune globulin sera.

The analyses were performed with the immune electrophoretic technique of *Grabar & Williams* (4) as modified by *Wadsworth & Hanson* (21). The comparative immune electrophoretic technique of *Wadsworth & Hanson* (21) was also employed.

RESULTS

Immune electrophoretic studies of milk with anti- γ -globulin sera gave one faint precipitate in the γ -globulin region and a dense precipitate mainly in the β -globulin area (Fig. 1a). With some immune sera two parallel lines instead of a single one were obtained in the β_2 -globulin region (Fig. 1b). The determinant specific for the precipitate in the γ globulin region in these experiments was called γ_a (Figs. 1a and b).

With some immune sera the line in the γ -globulin region did not cross the β_2 -precipitates but seemed to give a reaction of partial identity. This

* Kindly supplied by Docent I. Brattsten, Gothenburg and AB Kabri, Stockholm.

should mean that the line in the γ globulin region was formed by a determinant (called γ_b) which was also present on the substance forming one or both of the parallel precipitates in the β_2 globulin region (Fig 1 c). These immune sera obviously did not contain antibodies against the determinant γ_a .

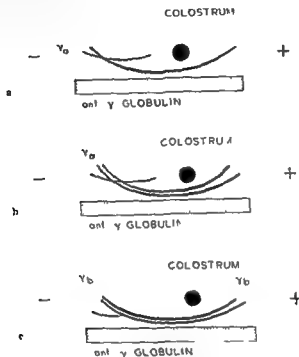


Fig 1 a b and c

Immune electrophoretic analysis of colostrum with various anti γ globulin immune sera. The determinants γ_a and γ_b are indicated.

Comparative immune electrophoretic studies of milk with the Bence Jones proteins of groups I and II showed that the BJ I gave a reaction of partial identity with that of the two parallel precipitates which was localized furthest from the antiserum basin (Fig 2 a). The BJ II in a similar experiment showed a reaction of partial interference with that of the two parallel precipitates in the β_2 globulin region which was localized nearest the immune serum basin (Fig 2 b). These experiments allowed designations of the antigenic determinants γ_c and γ_f (Fig 2 a) as well as γ_x and γ_h (fig 2 b).

A sheep anti blood plasma serum gave several lines in the β_2 globulin region in immune electrophoretic analyses of milk. One of these lines identified with serum β_{2M} globulin and all of the others which were parallel identified with serum γ globulin. One or possibly two of the latter lines did not identify with the two parallel lines formed by some

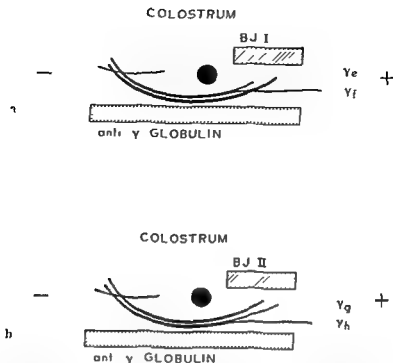


Fig 2

- a) Comparative immune electrophoretic analysis of electroseparated colostrum and a Bence Jones protein of group I (BJ I) by means of an anti γ globulin serum. The two separate determinants γ_e and γ_f are indicated
- b) Similar analysis as in a) but with a Bence Jones protein of group II (BJ II). The two determinants γ_g and γ_h are indicated

anti- γ -globulin sera (Fig 3 a). This was taken as evidence for the presence of one or possibly two γ -globulin determinants (called γ_d and γ_a in Fig 3 a) additional to those already demonstrated (Figs 1 and 2).

The presence of a determinant specific for serum β_{2A} -globulin could be demonstrated in the precipitate in the β_2 globulin region of immune electrophoretic analyses of milk with anti- γ -globulin or anti-immune globulin sera. In experiments where two parallel precipitates were formed the β_{2A} determinant was found in the precipitate nearest the immune serum basin (Fig 3 b).

Comparative analyses of milk with anti-colostrum sera and the aforementioned anti- γ globulin, anti-immune globulin and anti-blood plasma immune sera were performed. They showed that most of the anti-colostrum sera formed one dense precipitate mainly in the β_2 -globulin region which identified with the precipitates in which the earlier experiments had demonstrated the determinants called γ_b , γ_d , γ_e , γ_f , γ_g , γ_h and β_{2A} (Fig 4). With one anti-colostrum serum two parallel lines were obtained instead of the single dense β_2 -precipitate. Comparative studies showed that both of the Bence Jones proteins identified with one of these parallel precipitates (cf Figs 2 a and b). In addition this precipitate contained the β_{2A} determinant.

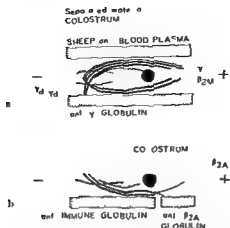


Fig 3

- a) Immune electrophoretic analysis of colostrum with a sheep anti blood plasma serum and an anti γ globulin serum. The experiment shows the presence of one or possibly two antigenic determinants (indicated γ_d and γ_d) against which antibodies are present in the sheep antiserum but not in the anti γ globulin serum. The precipitates identifying with serum γ globulin are indicated γ and the precipitate identifying with serum β_{2A} globulin is indicated β_{2A} .
- b) Comparative immune electrophoretic studies of milk with an anti serum immune globulin and an anti β_{2A} globulin immune serum. The precipitate formed by the determinant specific for β_{2A} globulin is indicated β_{2A} .

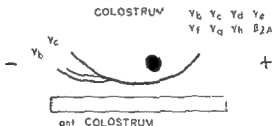


Fig 4

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pitate
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The anti colostrum sera also showed two precipitates in the γ -globulin region both of which identified with serum γ globulin and which gave a reaction of inhibition or of partial interference with the dense precipitate in the β_2 globulin area (Fig 4). One of the precipitates in the γ globulin region was formed by the determinant γ_h designated in an earlier experiment (Figs 1 c and 4). The determinant in the other precipitate was called γ_c (Fig 4). The determinants γ_b and γ_c should be present in the precipitate in the β_2 globulin area as well in that in the γ globulin region as judged from the reactions of partial identification or of inhibition (Fig 4).

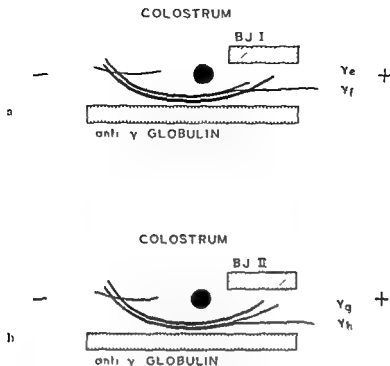


Fig 2

- a) Comparative immune electrophoretic analysis of electro-separated colostrum and a Bence Jones protein of group I (BJ I) by means of an anti γ globulin serum. The two separate determinants γ_e and γ_f are indicated
- b) Similar analysis as in a) but with a Bence Jones protein of group II (BJ II). The two determinants γ_g and γ_h are indicated

anti- γ -globulin sera (Fig 3 a). This was taken as evidence for the presence of one or possibly two γ -globulin determinants (called γ_d and γ_e in Fig 3 a) additional to those already demonstrated (Figs 1 and 2).

The presence of a determinant specific for serum β_{2A} -globulin could be demonstrated in the precipitate in the β_2 -globulin region of immune electrophoretic analyses of milk with anti- γ -globulin or anti-immune globulin sera. In experiments where two parallel precipitates were formed the β_{2A} determinant was found in the precipitate nearest the immune serum basin (Fig 3 b).

Comparative analyses of milk with anti-colostrum sera and the aforementioned anti γ -globulin, anti-immune globulin and anti-blood plasma immune sera were performed. They showed that most of the anti colostrum sera formed one dense precipitate mainly in the β_2 -globulin region which identified with the precipitates in which the earlier experiments had demonstrated the determinants called β_1 , γ_d , γ_e , β_1 , β_2 , γ_h and β_A (Fig 4). With one anti-colostrum serum two parallel lines were obtained instead of the single dense β_2 -precipitate. Comparative studies showed that both of the Bence Jones proteins identified with one of these parallel precipitates (cf Figs 2 a and b). In addition this precipitate contained the β_{2A} -determinant.

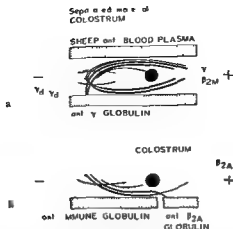


Fig 3

a) Immune electrophoretic analysis of colostrum with a sheep anti blood plasma serum and an anti γ globulin serum. The experiment shows the presence of one or possibly two antigenic determinants (indicated γ_d and γ_d) against which antibodies are present in the sheep antiserum but not in the anti γ globulin serum. The precipitates identifying with serum γ globulin are indicated γ and the precipitate identifying with serum β_{2M} globulin is indicated β_{2M} .

b) Comparative immune electrophoretic studies of milk with an anti serum immune globulin and an anti β_{2A} globulin immune serum. The precipitate formed by the determinant specific for β_{2A} globulin is indicated β_{2A} .

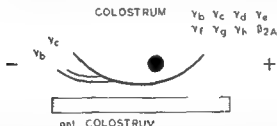


Fig 4

Immune electrophoretic analysis of milk with anti colostrum serum. Only the precipitates related to serum γ and β_{2A} globulins are shown in the figure. (The total pattern includes at least 22 precipitates). The determinants identified in the shown precipitates are indicated.

The anti colostrum serum also showed two precipitates in the γ globulin region both of which identified with serum γ globulin and which gave a reaction of inhibition or of partial interference with the dense precipitate in the β_2 globulin area (Fig 4). One of the precipitates in the γ globulin region was formed by the determinant γ_b designated in an earlier experiment (Figs 1 and 4). The determinant in the other precipitate was called γ_c (Fig 4). The determinants γ_b and γ_c should be present in the precipitate in the β_2 globulin area as well in that in the γ globulin region as judged from the reactions of partial identity or of inhibition (Fig 4).

DISCUSSION

To summarize the antigenic determinants of serum immune globulins that have been found in milk immune globulins there are at least eight determinants related to serum γ -globulin (called γ_a - γ_h) and one specific for serum β_A -globulin (indicated β_A) as well as one specific for serum β_{2M} -globulin (indicated β_{2M}). The γ_a , γ_b and γ_c are present on antigen or antigens localized in the region of slow γ -globulin (Fig 5). The determinants γ_b , γ_c , γ_d , γ_e , γ_f , γ_g , γ_h and β_A are present on antigen or antigens with a slightly smaller mobility than serum β_A -globulins. The presence of a milk antigen with the β_{2M} determinant is found in the same area.

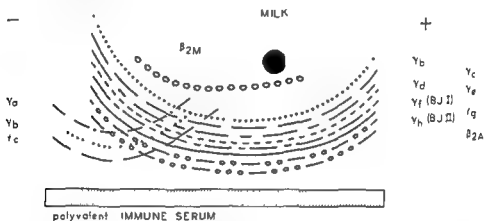


Fig 5

Schematic representation of the antigen mosaic of milk immune globulins. The diagram is arranged as an immune electrophoretic analysis where each line indicates the localization of a separate antigenic determinant. The determinants specific for serum β_{2A} and β_{2M} globulins are indicated β_{2A} and β_{2M} and those related to serum γ globulin are indicated γ_a - γ_h .

Thus the experiments show a large number of antigenic determinants common to the immune globulins of blood serum and milk. It is difficult to explain the precipitation patterns obtained if one assumes the presence of proteins in milk which are identical to the serum γ - and β_A -globulins. Instead there seem to exist in the milk a protein or possibly a group of proteins which have an electrophoretic mobility comparable to that of slow serum γ -globulin and which are provided with the antigenic determinants γ_a , γ_b , and γ_c . These milk proteins then would have antigenic determinants common to serum γ globulin but be antigenically deficient compared to this serum protein as they are lacking, i.e. the γ -globulin determinants γ_d - γ_h (cf Fig 5). It is suggested that this protein or group of proteins be called "milk γ -globulin".

The presence of one or more milk proteins of approximately β_2 mobility with γ -globulin determinants and another protein of identical mobility with the β_A determinant and possibly with one or more γ -globulin determinants as well might be considered as the background to the various precipitation patterns of β_2 -precipitates obtained with the dif-

ferent sera This does not explain, however, the precipitation patterns obtained where for example all the determinants γ_b - γ_h and β_{2A} were found in a single precipitate (Fig 4) or where some of these determinants were found in separate lines (Fig 2) In one experiment the determinants of the two different Bence Jones proteins were found in two separate precipitates, in another experiment both of these determinants were found in the same precipitate

Nor is the assumption of one milk protein provided with all of the determinants γ_b - γ_h as well as β_{2A} easily compatible with the experimental results (8, 9) It might be possible, however, to explain the obtained findings by supposition of a heterogeneous and immunologically complex group of proteins all of which should be primarily provided with the determinants γ_b , γ_h and β_{2A} Such a protein group might be called "milk β_{2A} globulin" This heterogeneous "milk β_{2A} -globulin" should then give rise to two or more parallel lines with some immune sera The same mechanism may be responsible for these parallel precipitates as for the separate parallel precipitates obtained in immune electrophoretic studies of serum γ globulin (3, 11) Serum γ -globulin has been characterized as a heterogeneous protein group, a "protein family" (11)

No proteins seem to exist in milk which are antigenically identical to the serum immune globulins but antibodies of the same specificity have been demonstrated in milk and serum (9) This may indicate that antibodies may have the same antibody specificity but may be antigenically different

SUMMARY

At least eight separate antigenic determinants present on normal serum γ -globulin as well as determinants specific for serum β_{2A} - and β_{2M} globulins have been demonstrated on milk proteins Three of the γ globulin determinants were found in immune electrophoretic precipitates in the region of slow γ -globulin Two of these together with the other five γ globulin determinants as well as the β_{2A} -determinant were found mainly in the β_2 globulin area It is suggested that in milk there exists a heterogeneous and immunologically complex group of proteins in which all of the proteins are primarily provided with the seven γ globulin as well as the β_{2A} -globulin determinants and with a mobility slightly smaller than that of serum β_2 -globulin This protein group has been designated "milk β_{2A} -globulin" Another heterogeneous milk protein group with a mobility of slow γ -globulin provided with three different γ -globulin determinants should be present as well This protein group has been called "milk γ -globulin"

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STUDIES ON C REACTIVE PROTEIN

1 *Demonstration of C Reactive Protein by Precipitation in Capillary Tubes and by Precipitation in Agar Gel*

By

LARS Å NILSSON and LARS Å HANSON

Received 24.11.61

Several methods have been applied to demonstrate the presence of C reactive protein (CRP) in human sera (2-6). One of the most common methods in the routine laboratory is the precipitation of CRP by means of specific rabbit immune sera in capillary tubes (17). CRP has also been studied by the double diffusion in gel method of Ouchterlony (3, 10, 13).

In the determinations of CRP in blood sera by the capillary tube method weak reactions are sometimes obtained. Since it is often difficult to decide whether or not these reactions are of a specific antigen-antibody nature we have studied such sera also with the Ouchterlony plate technique. The results obtained with the two methods have been compared.

MATERIAL AND METHODS

Human sera. The study included 166 samples taken from sera sent to the Bacteriological Laboratory.

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Immune sera. Rabbit CRP antisera (CRPA) commercially available from Schiefelbusch Ltd. were employed. Lot numbers were not given by the manufacturer, thus it is unknown whether or not the used samples originated from the same batch.

Methods. The determinations of CRP were performed in capillary tubes using the CRP antiserum according to the manufacturer's directions.

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RESULTS

The demonstration of C-reactive protein in the sera as shown by precipitation in capillary tubes and by precipitation in agar gel was compared (see table)

TABLE 1

	Precipitation in capillary tubes	Number of sera	Precipitation in agar gel		Positive in gel precipita- tion ¹
			Positive	Negative	
Patients	Positive	100	100	0	100
	Traces	28	26	2	93
	Negative	38	21	17	55
Blood donors	Positive	5	5	0	100
	Traces	14	10	4	71
	Negative	107	55	52	51
Controls	Negative	39	1	38	3

CRP was present in 100 of the sera from patients as found with the capillary tube technique, these sera were also positive when tested by precipitation in agar gel. Of the 28 sera showing only traces of CRP by tube precipitation 26 were positive with gel precipitation and two were negative. Finally, 38 of the sera from patients were negative by tube precipitation, but in 21 of these CRP was demonstrated by gel precipitation.

Of the sera from blood donors 5 were positive with both of the techniques. In 14 trace amounts of CRP were found by precipitation in capillary tubes, but only 10 of these were positive with the diffusion-in-gel method. Of the 107 sera from blood donors which were negative in capillary tubes 55 were positive by gel precipitation.

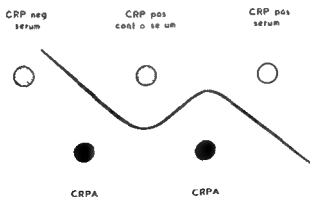


Fig 1

Schematic diagram of a double diffusion analyses showing the absence of CRP in one tested serum and the presence in another serum by means of a CRP positive control serum and a CRP antiserum (CRPA)

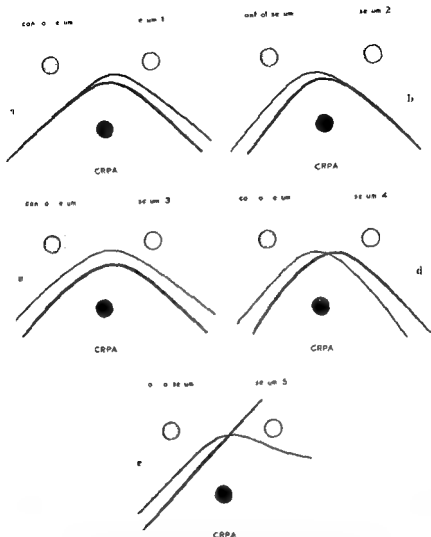


Fig. 2a-e

Schematic diagrams of comparative double diffusion analyses of various sera and control sera with CRPA. The control serum in *a* formed one line while those in *b, c, d* formed two with the CRPA employed.

In the control group of 39 individuals CRP was not demonstrable by the capillary tube method. All but one of these were also negative when tested by the diffusion in gel method.

In some of the experiments the CRP control as well as the tested sera give one precipitation line with the CRPA (Fig. 1). In several experiments, however, two precipitates were obtained and in some instances a third faint line was observed. In the comparative studies of the reference sera and the analyzed sera different precipitation patterns

were obtained: A single line formed in some of the reference spectra identified with one or in some cases with two lines formed by the tested sera (Figs 1 and 2 a) The two lines formed in some of the reference spectra identified with one or with each one of the two lines formed by some of the examined sera (Figs 2 b and c) In the latter case a crossing was sometimes obtained between the two precipitation lines (Fig 2 d) In several experiments a reaction of identity was observed only for one of the precipitation lines in the reference spectrum (Fig 2 e) The precipitate nearest the immune serum basin showed a reaction of non-identity and crossed the second precipitate in all of the latter analyses

DISCUSSION

The occurrence of the C-reactive protein (CRP) in human sera in relation to certain diseases has been found to be of some diagnostic help (1, 4, 11, 12) Several techniques have been used for the demonstration of CRP, among others the precipitation in agar (3, 10, 13) Using this technique it was possible to show the specific nature of most of the weak reactions (< 1 mm) obtained by precipitation in capillary tubes A few of the weak reactions, however, were obviously of a non-specific nature These nonspecific precipitates may possibly have been caused by some degree of denaturation of the used material Precipitates equal to more than 1 mm in length in the capillary tubes were always specific CRP-CRPA reactions as verified by the diffusion-in-gel analyses

About 50 per cent of the sera which were negative with the capillary tube method were shown to contain CRP by means of the diffusion-in-gel technique To control these findings we analyzed sera from blood donors with similar results This confirmation gave rise to the question of whether CRP is normally present in human sera as illustrated by a precipitation-in-gel technique or whether this sensitive method reveals the presence of small amounts of CRP caused by clinical or subclinical conditions To pursue this inquiry we tested sera from individuals where we had proper anamnestic and clinical evidence of good health and which had no CRP demonstrable by the capillary tube method Only in one of these sera could we demonstrate any CRP by the diffusion in-gel method This might indicate that CRP cannot be shown in sera from healthy individuals by the Ouchterlony method Detailed clinical and anamnestic information about the blood donors are lacking and the presence of CRP in more than half of the number of the sera from blood donors might have been due to e.g. light or subclinical infections Determination of CRP by the capillary tube method in 4803 sera from blood donors gave a positive reaction in 91 per cent (8) At that time trace reactions were counted as positive

As illustrated by our findings in studies of C-reactive protein the double diffusion-in gel method is advantageous due to its specificity and great sensitivity A certain heterogeneity of the C-reactive protein has

been discussed by some authors (7,9,13,15) and has been demonstrated by absorption studies with pneumococcal polysaccharides (2). An evidence for a heterogeneity was obtained in comparative analyses where crossings between two of the precipitation lines occasionally formed by the CRP-CRPA system was observed (Figs 2d and e). Further evidence for such a heterogeneity has been obtained in recent comparative diffusion in gel studies of CRP and polysaccharides prepared from some pneumococcal strains (12) confirming the results of Hedlund (2).

SUMMARY

The results of demonstration of C reactive protein in human sera by precipitation in capillary tubes and by precipitation in agar gel have been compared.

- (1) CRP demonstrable by the capillary tube method (prec > 1 mm) was always demonstrable by gel precipitation.
- (2) Weak reactions obtained by the capillary tube method were mostly found to correspond to specific precipitates by the diffusion in gel method. Some of these weak reactions were probably nonspecific, however.
- (3) CRP was found by the precipitation in gel technique in several sera which were negative with the capillary tube method.
- (4) None of the methods showed CRP in sera from healthy individuals.

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DISCUSSION

The occurrence of the C-reactive protein (CRP) in human sera in relation to certain diseases has been found to be of some diagnostic help (1, 4, 11, 12). Several techniques have been used for the demonstration of CRP, among others the precipitation in agar (3, 10, 13). Using this technique it was possible to show the specific nature of most of the weak reactions (< 1 mm) obtained by precipitation in capillary tubes. A few of the weak reactions, however, were obviously of a non-specific nature. These nonspecific precipitates may possibly have been caused by some degree of denaturation of the used material. Precipitates equal to more than 1 mm in length in the capillary tubes were always specific CRP-CRPA reactions as verified by the diffusion-in gel analyses.

About 50 per cent of the sera which were negative with the capillary tube method were shown to contain CRP by means of the diffusion-in gel technique. To control these findings we analyzed sera from blood donors with similar results. This confirmation gave rise to the question of whether CRP is normally present in human sera as illustrated by a precipitation-in gel technique or whether this sensitive method reveals the presence of small amounts of CRP caused by clinical or subclinical conditions. To pursue this inquiry we tested sera from individuals where we had proper anamnestic and clinical evidence of good health and which had no CRP demonstrable by the capillary tube method. Only in one of these sera could we demonstrate any CRP by the diffusion-in gel method. This might indicate that CRP cannot be shown in sera from healthy individuals by the Ouchterlony method. Detailed clinical and anamnestic information about the blood donors are lacking and the presence of CRP in more than half of the number of the sera from blood donors might have been due to e.g. light or subclinical infections. Determination of CRP by the capillary tube method in 4803 sera from blood donors gave a positive reaction in 9.1 per cent (8). At that time trace reactions were counted as positive.

As illustrated by our findings in studies of C-reactive protein the double diffusion-in-gel method is advantageous due to its specificity and great sensitivity. A certain heterogeneity of the C-reactive protein has

TRANSACTIONS OF THE MEDICAL MICROBIOLOGY DIVISION OF THE SWEDISH MEDICAL SOCIETY

Meeting December 1, 1961

NEW ASPECTS ON TISSUE ANTIGENS

Some New Techniques for the Detection of Tissue Antigens

Fspmark A & Fagraeus Astrid The National Bacteriological Laboratory, Stockholm
STUDIES ON CELL SURFACE ANTIGENS BY THE MIXED HAEM
ADSORPTION TECHNIQUE

This method (earlier outlined for some viral antigens—*Nature* 190 370 1961) is essentially an antiglobulin test applied to cells in living monolayer cultures. The binding between cell antigens and corresponding antibodies of an applied antiserum is indicated by adsorption to the tissue of added indicator sheep erythrocytes. The reactivity of these cells is effected by coating them with an antiglobulin directed against the species which produced the anti tissue antibody.

To achieve this antiglobulin coating it was necessary first to have the corresponding globulin fixed to erythrocytes using e.g. rabbit amboceptor serum (rabbit system) or human or horse immune sera against staphylococcal or diphtherial toxoid interacting with tanned erythrocytes carrying the respective toxoids (human and horse system).

In culture bottles with the tissue under study in confluent sheets and covered with 0.7% per cent isotonic agar the antitissue sera were applied in serial dilutions to filter paper discs on the agar. After a diffusion time of 1-2 days the agar was poured off and the cultures washed once. Addition of indicator cells gave pronounced haemadsorption zones. The size of zones were closely related to antiserum concentration.

Tests with unadsorbed sera did not reveal significant antigenic differences between several human tissues. There was strong cross reaction between human and monkey tissue and weaker although pronounced reaction between human and bovine tissue. Minor interspecies differences could be elucidated through cross adsorption of antisera with cells. Similar work is in progress on intraspecies differences.

Titers of antihuman and antihorse tissue sera were significantly decreased by adsorption with human AB blood. No antigenic relation seemed to be present between serum components and cell surface antigens.

Möller G Department of Tumour Biology, Karolinska Institutet, Stockholm
DEMONSTRATION OF MOUSE ISOANTIGENS AT THE CELLULAR LEVEL BY
THE FLUORESCENT ANTIBODY TECHNIQUE

The fluorescent antibody technique has been applied for the demonstration of mouse is antigens at the cellular level. Specific reactions were obtained by the

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on spleen cells with cytotoxic tests. No reactivity could be demonstrated with any of the techniques during the first three days after birth. Adult reactivity was reached after 6-10 days both with red blood cells and with spleen cells. Absorption experiments carried out *in vivo* indicated that newborn mice possessed isoantigens during the first three days after birth. *In vitro* absorptions demonstrated that spleen, liver and kidney cells were antigenic while no significant antigenicity was found on erythrocytes less than three days old. Since spleen cells were found to be antigenic their absence of reactivity to humoral antibodies cannot be ascribed to an absence of antigenic receptors.

With the fluorescent antibody technique it was found that all spleen cells from mice less than three days old were antigenic. The frequency of antigenic cells in embryonic liver cells diminished with age. No cells capable of reacting with humoral isoantibodies were found in liver cell suspensions from 15 day old embryos.

A quantitative *in vitro* absorption technique was worked out and applied to the study on the antigenicity of newborn spleen cells at various ages. It was found that the concentration of isoantigenic surface receptors increased with age and reached adult level after 11 days. The same technique was used for the study of the antigenicity of a variety of normal and neoplastic cells. It was found that different cell types varied much as regards their ability to absorb isoantibodies. High correlation coefficients were found between the concentration of isoantigenic surface receptors and the cytotoxic titer.

Mårtenson L. Institute of Bacteriology, University of Lund, Lund. DISTRIBUTION OF Gm SPECIFICITIES AMONG THE GAMMA GLOBULIN MOLECULES

24 M-components ("abnormal" serum globulins in myeloma and related diseases) which immunoelectrophoretically were of the γ type were studied for the specificities Gm(a), Gm(b) and Gm(x). 7 were Gm(a+b-), 2 Gm(a-b+), 9 Gm(a-b-), 1 Gm(a±b+) and 5 Gm(a+b-). Type Gm(a+b+) was not encountered. This frequency distribution differs grossly from the one found in normal sera. But the frequency distribution of the different Gm types of the normal γ globulin in the same sera did not appear to differ from the one in normal sera. Only 3 of the M-components were classified regarding Gm(x). One of these was Gm(a+b-x+) and two were Gm(a-b+x-). Some of the M-components were much more inhibitory than the γ globulin in Gm(+). normal sera. The findings are compatible with the hypothesis that Gm(a) and Gm(b) specificities in normal sera are carried by different molecules. Among 111 M-components of β_2 type and three of β_2 type the Gm(a), (b) or (x) specificity could not be demonstrated in any case.

Anti D antibodies of Gm(a+b+) sera were used to coat 11 Rh+ red cells. Judging from the agglutination patterns obtained with a number of rheumatoid arthritic sera these anti D antibodies often lacked Gm(b) specificity and in one case Gm(a) specificity.

All 11 antibodies carrying Gm(a) specificity are more frequently encountered than anti D antibodies carrying Gm(b) specificity. This is in accord with the finding that Gm(a+) M-components were more common than Gm(b+) in normal sera, on the other hand the type Gm(b+) is more common than the type Gm(a).

When two different incomplete blood group antibodies (anti D and anti Fy(a)) occurring in one and the same serum had been used to coat the appropriate cells these cells gave qualitatively different agglutination patterns with a number of rheumatoid arthritic sera. The most probable explanation of this is that the two antibodies differed with respect to Gm specificities.

indirect or sandwich technique with a variety of living normal and neoplastic cells. Isoantigens of the H 2 system and of other systems could also be demonstrated and appeared to be localized to the cell membrane. As far as the H 2 system was concerned the membrane localization could be confirmed on histological sections.

Different types of nonspecific staining reactions have been identified and described. Pinocytosis and cell injury were followed by such reactions as were morphologically distinguishable from the specific 'ring' reaction and with a view to pinocytosis were easily avoided by reducing the incubation time. In addition a non specific 'ring' reaction could be seen in a small proportion of bone marrow and lymph node cells but in no other cell types studied. It was found that embryonic cells lacked this staining reaction completely and that the frequency of positive control cells showing the ring reaction increased with age in newborn mice and reached adult level in seven days. The possible nature of this reaction was discussed.

Hogman C I, Fjellstrom K E & Kullander J. The Department of Virus Research, Karolinska Institutet, Stockholm and the Department of Clinical Chemistry, University Hospital, Uppsala.
STUDIES ON BLOOD GROUP SPECIFIC SURFACE ANTIGENS OF HUMAN FOETAL TISSUE CELLS BY SEROLOGICAL ADHESION TECHNIQUE

The fixation of blood groups A and B antibodies on the surface of cultured human foetal kidney and lung cells can be demonstrated by mixed agglutination (MA). The reaction is indicated by the specific adhesion of red cells carrying an antigen corresponding to that of tissue cells.

However, in the presence of a thermolabile factor present in normal human sera, red cells devoid of the homologous antigen (e.g. human group O erythrocytes) are also adhered to kidney and lung cells. To test the mechanism of this reaction it was performed in two steps. In the first step a thermostable substance is fixed which can be demonstrated to consist of blood group A or B antibodies. In the second step the thermolabile accessory factor is acting. The mechanism seems to be very similar to the one seen in serological adhesion (SA) (Bact Rev 21:30, 1957).

Blood group A antibodies in human sera were separated in 7 S and 19 S fractions by chromatography on DEAE Sephadex and by gel filtration on Sephadex G 200. In most experiments the 7 S fractions did not give MA but caused SA. The 19 S fractions reacted by both methods.

The thermolabile accessory factor and its relation to the complement system was tested by complement reagents. The factor consisted of several components but not of the whole human complement. The experiments strongly suggested that the reacting factors were the 11 S component C1 and C4. There was no evidence that C3, C3 properdin or β_{1C} took part in the reaction.

The Development and Specificity of Tissue Antigens in Relation to Tolerance and Genetics

Uller G. Department of Tumor Biology, Karolinska Institutet, Stockholm.
STUDIES ON THE DEVELOPMENT OF THE ISOANTIGENS OF THE H 2 SYSTEM IN NEWBORN AND EMBRYONIC MICE

The development of cellular isoantigens of the H 2 system was studied on red blood cells from newborn mice using the agglutination and haemolysis techniques and

(2) Human colonic cells (from foetuses) were tested against peripheral lymphocytes from patients suffering from ulcerative colitis and from patients suffering from other diseases. Exposure of labelled (^{32}P) colonic cells to lymphocytes from ulcerative colitis patients caused a 2-3 times greater loss of lipids, nucleotides and RNA from the colonic cells than did exposure to lymphocytes from patients suffering from other diseases. Serum from patients with ulcerative colitis seemed not to exert any cytotoxic effect on human colonic cells *in vitro*.

The results also indicated that the cytotoxic process requires the presence of complement.

Meeting December 2, 1961

FRFEL PAPERS VIROLOGY

Antlings L O. The National Bacteriological Laboratory, Stockholm. LYSIS FROM WITHOUT OR WITHIN WITH THE O-1 PHAGE SPECIFIC TO THE GENUS *SALMONELLA*.

When undiluted phage suspensions (about 10^7 particles/ml) were used for spot test in subcultures from desoxycholate-tetracycline or Fud agar in routine conditions, positive phage reactions occurred in 98.8 per cent of the serologically verified *Salmonella* cultures (521) corresponding to 12 per cent positive reactions with non-*Salmonella*s (63a). Only 75.4 per cent of 215 *Salmonella* cultures sent in for typing at the reference laboratory and representing 57 different serotypes were phage positive.

All these 215 cultures were retested in spot tests using an undiluted lysate of high titer (2×10^{11} particles/ml) 4 hours broth cultures and agar plates. Positive reaction was now obtained in 214 cultures (99.5 per cent). Growth curves revealed a prolonged lag phase and restricted growth of the remaining strain in the presence of phage. Only 2 of 2260 *S. paratyphi* B and *S. typhimurium* cultures were negative when tested with the strong preparation.

Tenfold dilutions of #1 were tested on the 215 *Salmonella* cultures. Low concentrations caused late plaques in 51 per cent of the cultures. As low concentrations as 2×10^4 particles/ml caused confluent lysis of these cultures which were evidently lysed from within.

Late plaques were observed in the remaining 49 per cent of the cultures. A reaction resembling confluent lysis was caused only by high phage concentrations $> 2 \times 10^6$. The number of surviving bacteria and adsorbed phages showed that only high phage:bacterium ratios caused the reaction suggesting a lysis from without.

One step growth curves on the propagating strain revealed a latent period of 25 minutes and a burst size of 2×10^6 .

Arshady A. & Min H. The Department of Virus Research, Karolinska Institutet, Stockholm. ISOLATION AND IDENTIFICATION OF A SYNCYTIUM-FORMING VIRUS.

In the course of an extensive study on the etiology of infectious hepatitis, an apparently viral cytopathogenic effect in an established human embryo lung cell strain Lu 106 was recently encountered by one of us (H.A.). Although it soon

The Reaction of Tissue Antigens with Antibodies or Antibody-like Factors

Jonsson J & Fagraeus Astrid The National Bacteriological Laboratories Stockholm
STUDIES ON ANTINUCLEAR FACTORS WITH THE MIXED HAEM
ADSORPTION TECHNIQUE

At least three different antinuclear factors are known to occur in sera from patients with systemic lupus erythematosus (SLE)

the LE cell factor probably reactive to native nucleoprotein

the anti DNA factor found in about 50 per cent of the cases and so far exclusively in SLE

one or more factors reactive to nuclear substances which are soluble in physiological buffers

The present authors have tried to apply the mixed haemadsorption technique to these serological systems

The procedure conformed to the one used by *Fspmarl & Fagraeus* although in the present work the antigens were dried on the bottom of Petri dishes and fixed with acetone or methanol

The antigenic preparations used were derived from a distilled water nuclear extract of calf thymus

Among the SLE sera examined two main partially overlapping groups could be discerned by way of their different reactivity to two different antigenic preparations

Some evidence was obtained showing that one preparation was antigenic to the anti DNA factor while the activity of the other was due to another material soluble in physiologic buffered saline

Attempts to demonstrate the LE cell factor in the same way were unsuccessful

18 SLE sera have been thoroughly examined 9 reacted with the DNA antigen and 11 with the soluble antigen while 15 gave positive LE cell preparations However when the mixed haemadsorption tests were used together their combined diagnostic capacity equalled the one of the LE cell test i.e. positive reactions were obtained with 15 sera

Broberger O Perlmann P & Klein E The Wenner Gren Institute Stockholm University Stockholm A QUANTITATIVE METHOD FOR AN ESTIMATION OF THE CYTOTOXIC EFFECT EXERCISED BY CELLULAR ANTIBODIES ON TARGET CELLS IN VITRO WAS DESCRIBED

Largely cells labelled with radioactive phosphorus or carbon (^{32}P or ^{14}C) were incubated with lymphocytes from immunized or non immunized individuals in or without the presence of complement By fractionation of cells and incubation medium and subsequent determination of the radioactivity in the different fractions it was possible to get an idea about the release of nucleotides RNA lipids and protein from the labelled cells

Two immunological systems were tested

1 MCFM tumour cells from C3H mice were tested against lymphnode cells from C57 mice immunized with tissue from C3H Incubation of labelled (^{14}C) tumour cells with lymphnode cells from immunized C57 mice caused a 1.6 times greater loss of RNA nucleotides and protein than did incubation with lymphnode cells from non immunized C57 mice Immune serum was shown to be non toxic when tested with this method

Readings were taken for NDV after incubation for 3-6 days and for the slower growing SV₅ plaques after incubation for 6-10 days at 36° C.

The correlation of plaque counts to dilution of inoculum was satisfactory for both viruses which applies also to the reproducibility of titers on different occasions.

In SV₅ infected cultures where tissue was accidentally or voluntarily removed from the glass at harvest addition of erythrocytes resulted in haemadsorption plaques on the glass similar in size and number to those found in corresponding bottles with intact tissue.

Adsorption of SV₅ virus to tissue was quick if a small volume of inocula were used. After 2 hours at room temperature no further adsorption seemed to take place. Almost 50 per cent of this quantity was absorbed in the first 15 minutes. Elevation of temperature increased the adsorption rate markedly.

By application of filter paper discs soaked in immun sera to the agar in heavily infected cultures the neutralization zones obtained were seen as circular defects in the confluent haemadsorption at the final reading. The diameter of these zones approximately indicated the antibody content in the serum. Serum samples were screened from different batches of monkeys by testing each serum in one dilution (1:10) in such disc tests by which it could be estimated whether a monkey batch was heavily infected or relatively free. Antibodies were found only occasionally in individuals engaged in animal work and in humans without monkey contacts. Titers of about 1:100 were found in human gamma globulin preparations.

Hermansson S & Westman J Institutes of Virology and Anatomy Uppsala University Uppsala MORPHOLOGY OF PARAINFLUENZA VIRUS TYPE 3

To be published in J Ultrastruct Res

Philipson L Institute of Virology Uppsala University Uppsala TITRATION OF ADENOVIRUS BY THE FLUORESCENT ANTIBODY TECHNIQUE

Published in Virology 10: 263 1961

Kjellen I Malmö Allmänna Sjukhus Malmö EFFECT OF DNA ANTIMETABOLITES ON CELL PROLIFERATION AND ADENOVIRUS SYNTHESIS

During the past year many agents have been tested for their ability to inhibit the growth of malignant tumours. Most of these substances are structural analogs of pyrimidines and purines which form part of the nucleic acids. Their biological effect is probably due to their interference with the natural bases of the nucleic acids and they have therefore also become a tool in virological research.

The following work deals with the influence exerted by 5-Fluorodeoxyuridine (FU_{DR}), 5-Bromodeoxyuridine (BU_{DR}) and aminopterin on 1) the proliferation of a human cell strain (MAS 4) and 2) the multiplication of adenovirus type 5 (143 strain).

Cell division was followed using the Puck technique i.e. about 100 single cells seeded in a Petri Dish were studied over an eight-day period during which the number of newly developed cells per colony were counted. 0.5 per cent lactalbumin hydrolysate in Earle's salt solution enriched with 10 per cent human serum was used as growth medium.

Virus proliferation was followed by one step growth experiments. Titration of virus was performed by the plaque technique.

became apparent that the transmissible agent responsible for this effect represented a pick up an identification was attempted

Cytopathic changes notably formation of large multinucleated syncytia appear within a few days in Lu 106 HeLa, Detroit 6 tissue cultures inoculated with undiluted tissue culture material The maximal virus titers obtained range between 10^4 to 10^5 per ml The virus is destroyed by ether treatment and is fairly thermolabile A striking haemadsorption can be shown in inoculated cultures in contrast to normal cultures and the virus possesses haemagglutinating properties similar to those characteristic for myxoviruses The haemagglutinin readily elutes and the receptors on the erythrocytes are destroyed by treatment with periodate and receptor destroying enzyme Analysis by centrifugation in a sucrose density gradient shows the conversion into a free small haemagglutinin after treatment with ether It also suggests the physical properties of the virus to be similar to those of parotitis and parainfluenza viruses rather than to those of influenza viruses In haemagglutination-inhibition tests cross reactions were obtained with antisera against parotitis virus and three previously identified simian myxoviruses, including SV5 A corresponding inhibition of two of the simian myxoviruses but not of parotitis virus was shown by a specific antiserum against the virus here investigated

Vorrbj F The Department of Virus Research Karolinska Institutet, Stockholm
HAEMAGGLUTINATION BY MEASLES VIRUS

Analyses of measles tissue culture material show a comparatively low ratio of infectious to haemagglutinating titers This suggests the presence of either large numbers of noninfectious virus particles or a separate haemagglutinin

It was shown that the infectious particles adsorb to the erythrocytes At the same time centrifugation experiments indicated the presence of a smaller noninfectious haemagglutinin This was verified by centrifugation of virus material in a continuous sucrose density gradient One fast sedimenting fraction carrying all the infectivity and a small part of the haemagglutinating activity as well as a slower fraction which was noninfectious although responsible for the major part of the haemagglutinating activity was found By separation of the two haemagglutinins it was possible to study their interrelationship The large one was converted into small haemagglutinin by treatment with ether or after thermal degradation at 37°C with simultaneous loss of infectivity Furthermore analysis of three different virus cell systems showed a positive correlation between the rate of virus reproduction and the proportion and absolute amount of large haemagglutinin in the tissue culture fluid This suggests that the large haemagglutinin which possesses the infectious properties is probably the primary product A thermal degradation of the virus particles causes appearance of the noninfectious small haemagglutinin which thus is a secondary product

Espmark A The National Bacteriological Laboratory Stockholm
PLAQUE STUDIES ON MYXOVIRUSES

Haemadsorption plaques were produced in bottle cultures of monkey cells infected with NDV or simian myxovirus (SV5) Before reading the 0.75 per cent agar overlay is easily poured out after some 10 cc of saline has been added One per cent suspension of preferably human chicken (for NDV) or sheep (for SV5) erythrocytes were added and plaques counted after 10 min at room temperature

Wallerstrom A Institute of Bacteriology University of Lund Lund GROUPING STREPTOCOCCI BY BIOCHEMICAL METHODS

Three methods for distinguishing group A streptococci from other β haemolytic streptococci has been investigated sensitivity to bacitracin stimulation of haemolysis by nucleate preparations and haemolysis inhibition by glucose The glucose inhibition of haemolysis is certainly due to a suppressed development of streptolysin S as serum extracts from A streptococci grown in glucose broth in the author's experiments gave no haemolysis while the same strains when grown in plain broth or lactose containing media gave haemolysis to full extent Addition of glucose to the serum extracts did not alter haemolysis—The tests mentioned may be performed at the same time as a triple test The author's material consist of 140 streptococcal strains 72 belonging to group A G-6 the remainder representing all other serological groups except F and Q The test was performed with bacitracin discs containing 0.2 IU/ml glucose discs containing a 10 per cent solution and nucleate tablets containing 30-60 per cent sodium nucleate All of the 53 A strains in the collection were sensitive to bacitracin and had their haemolysis stimulated by nucleate and inhibited by glucose i.e. triple test positive One single G strain showed the same pattern the remaining 86 non A strains were bacitracin resistant or failed to react to glucose or nucleate (triple test negative) It is concluded that the combination bacitracin sensitivity nucleate stimulation and glucose inhibition is sufficiently uncommon outside the A group to make the triple test useful as a screen test for distinguishing A streptococci from non A strains

Andersen H.J. Hansson I. A. & Nilsson L. A. The Department of Pediatrics and the Department of Bacteriology University of Gothenburg Gothenburg THE PRESENCE OF C-REACTIVE PROTEIN IN THE NEONATAL PERIOD CORRELATED TO CLINICAL CONDITIONS

The quantity of C-reactive protein (CRP) in mothers will increase towards the end of pregnancy Only rarely CRP has been found in sera from the umbilical cord indicating that CRP does not pass the placenta Still CRP is demonstrable in sera from children one or a few days old Thus the newborn infant can form CRP It has been our intention to see whether this formation might be related to clinical conditions in the newborn child

Sera from 40 children and their mothers were analyzed for the presence of CRP by means of precipitation in capillary tubes and in agar gel The children were followed by daily clinical examinations and blood was taken at birth on the first second and fifth day

Confirming earlier investigations we found from only a few cases

per cent of the cases otherwise presenting no signs of disease indicates that the CRP test is inapplicable in clinical work during the first week of life This is further illustrated by the absence of CRP in some of the cases presenting various signs of disease

The results are given as the concentration of the analogs necessary to inhibit
 1 more than two cell divisions 2 any yield of virus production

	FUDR	BUDR	Aminopterin
1 Cell division inhibited by	40 μ g/ml	20 μ g/ml	No effect up to 40 μ g/ml
2 Virus production inhibited by	≤ 2 μ g/ml	30 μ g/ml	≤ 20 μ g/ml

By adding the FUDR to the cultures at varying times after virus infection it was shown that the viral DNA synthesis preceded the formation of infectious virus particles by about 10 hours in this system

In contradistinction to the treatment with FUDR or Aminopterin the BUDR treatment did not protect virus infected cells from cytopathic degeneration nor did it prevent the development of complement fixing antigens The results will be given in detail in another place

FREE PAPERS BACTERIOLOGY

Lindahl T & Heden C G Institute of Bacteriology Karolinska Institutet Stockholm CHANGES IN BIRTHRATE OF VICE TREATED WITH CLPAVAG PRODUCTS OF DNA

Edebo L Institute of Bacteriology Uppsala University Uppsala THE INACTIVATION OF BACTERIA BY ELECTRIC DISCHARGE IN WATER

Danielsson B Institute of Bacteriology Uppsala University Uppsala ON THE SPECIFICITY OF THE REACTION OF GONOCOCCI WITH FLUORESCENT ANTIBODIES

Gonococcal anti sera were produced by immunizing rabbits with formalin killed or heat killed organisms Six to eight strains of gonococci were pooled and used for the immunization By the conventional techniques of agglutination and complement fixation the above sera gave results of varying strengths The globulin fraction of six different gonococcal anti sera were conjugated with fluorescein isothiocyanate (FITC) and in some cases with lysamine rhodamine B200 (RB 200) The unbound FITC and RB200 were removed by passing the conjugates through a sephadex column (Sephadex G 25) FITC conjugated globulins gave brilliant staining reactions with gonococci while RB200 conjugates produced definitely weaker staining reactions By testing the conjugates against various gram negative and gram positive cocci it was found that relatively brilliant staining reactions were produced with meningococci belonging to sero groups A and B After absorbing the conjugates with meningococci of groups A and B this crossreaction was removed without appreciably reducing the reaction with gonococci Weak staining reactions were produced however with *Neisseria catarrhalis* and streptococci group G About 80 gonococcal strains isolated in Uppsala and Stockholm were tested with the six different gonococcal anti globulins conjugated with FITC They all produced brilliant staining reactions with the gonococci anti globulins

TRICHINOSIS IN SWEDEN IN 1961

Ringert O Lundbäck H & Zetterberg B The National Bacteriological Laboratory, Stockholm **EPIDEMIOLOGY**

Outbreaks of trichinosis are rare in Sweden. In 1937 33 cases were reported from Lindsberg and in 1946 35 cases appeared in Borås (Wird K 1946).

During the second week of July 1961 an outbreak of trichinosis occurred in the city of Karlskrona and the eastern part of the county of Blekinge on the south east coast of Sweden. The epidemic had its maximum around the 23rd of July. Only a few cases appeared after August 1st. Totally 338 cases are known: 52 per cent men and 48 per cent women. Most of the patients were 15-60 years old while only a few cases were children and people over 60.

Samples were collected from 276 cases. Details concerning the clinical picture and the consumption of food were received by questionnaire.

For epidemiological reasons a certain sausage smoked for 3 days at 30° C and containing 40 per cent pork was suspected to be the vehicle. A significant difference in frequency of positive serological reactions was found between patients who had consumed this particular product and those who had not. No significant difference in this respect was for 67 other pork products.

The majority of the cases were found within the distribution area of the slaughter house producing this sausage. The products were distributed by cars covering different areas on different days of the week. The relationship between these areas and the distribution of the cases was studied. It is considered probable that the distribution took place on the 27th and 28th of June. It is possible that some of the cases were due to other pork products but only relatively few cases are likely to have been infected in this way.

Hederstedt B Kallings L O Lundbäck H & Ringert O The National Bacteriological Laboratory, Stockholm **SERODIAGNOSIS**

The present outbreak offered an exceptional opportunity for comparing the sensitivity and specificity of certain serological tests on a well defined material. About 700 blood specimens from 276 patients and 50 healthy subjects were tested with the complement fixation reaction and the Roth microprecipitation reaction with living trichinella larvae. Skin test was performed on 142 patients and healthy subjects.

The cf reaction was positive in 39 per cent of the cases from the outbreak whereas the precipitin reaction was positive in 72 per cent and weakly positive in 19 per cent. The skin test was positive in 70 per cent. Among the healthy subjects one was positive in the cf test, 16 were weak Roth positive and 3 were skin test positive.

Forty-seven cases showing an increase of the cf titer of two dilution steps or more were all positive in the precipitin reaction. In a group of 53 patients demonstrating an increase in the strength of the precipitin reaction considered to be significant about 50 per cent were negative in the cf reaction. 81 and 67 per cent of the cf positive and negative cases respectively were skin test positive. There were no major differences between the groups as to the frequency of the typical symptoms or of the consumption of the sausage suspected to be the vehicle.

In the groups with negative cf reactions and moderate weak or negative precipitin reactions there was a tendency to lower frequency of positive skin tests, of the typical symptoms and of the consumption of the sausage. In the serologically negative group 37 per cent of the skin tests were positive.

Lund Fbba Lyl = E. & Sourander P The Virological Department of the Municipal Laboratories of Gothenburg and the Bacteriological and Pathological Departments University of Gothenburg Gothenburg PRELIMINARY STUDIES ON HOST CELL—TOXOPLASMA PARASITE INTERRELATIONS

Observations on the multiplication of *Toxoplasma gondii* in different cell types of human origin were described in previous reports. These studies gave a basis for further investigations of factors influencing the parasite—cell interrelationship.

In order to see whether inhibition of mitotic activity affected the parasite propagation irradiation of cell cultures and of parasites was performed. Irradiation with 30 000 to 120 000 rad by means of Co 60 was used.

The irradiation inhibited the mitotic activity of the parasites. The antigenic properties of the parasites studied by dye tests were unaffected. Irradiated host cells without mitotic capacity supported multiplication of parasites just as did the non irradiated cells.

In a second series of experiments the influence of the nutritional state of host cells on the multiplication rate of parasites was studied. The serum concentration of the cell culture medium was varied. Addition of dye test negative human serum in increasing concentrations slowed down the multiplication rate of the parasites.

The experiments have thus shown that mitotic capacity of the host cell is not necessarily required for the support of parasite multiplication. It seems also possible to influence parasite multiplication by altering the composition of the cell culture medium.

Strannegård O The Virological Department of the Municipal Laboratory and the Department of Bacteriology University of Gothenburg Gothenburg
PRECIPITINS IN TOXOPLASMOSIS AND THEIR RELATIONSHIP TO THE DYE TEST AND COMPLEMENT FIXING ANTIBODIES

By means of gel diffusion methods toxoplasma precipitating antibodies have been demonstrated in sera from patients and rabbits with toxoplasmosis. At least two identical precipitating antibody components were demonstrated in human and rabbit antisera. Sixtyseven human sera were tested for the presence of toxoplasma antibodies. Apparently there was no relationship between the number of precipitating antibody components and the dye test titers. The precipitin test was considerably less sensitive than the dye test. The concentration of complement fixing antibodies was in fair correlation to the number of precipitating components but in some cases no precipitins could be demonstrated in spite of rather high complement fixation test titers. Precipitins were demonstrated in human sera more than two years after the onset of the disease.

The development of antibody response during the course of an experimentally induced toxoplasmosis was studied in rabbits. The number of precipitating components in sera increased slowly to peak levels about three months following infection with living toxoplasma parasites, i.e. about two months after the maximum dye test and complement fixation test titers were demonstrated. All three types of antibodies persisted at almost unchanged concentrations for at least fifteen months.

Electrophoretic experiments showed that dye test and complement fixing antibodies of human and rabbit sera as well as toxoplasma precipitins of rabbit sera had a mobility similar to human blood serum β_2 globulins whereas precipitins of human sera seemed to be γ globulins with small mobility.

TRICHINOSIS IN SWEDEN IN 1961

Ringertz O Lundbeck H & Zetterberg B The National Bacteriological Laboratory
Stockholm EPIDEMIOLOGY

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Hellerstedt B Kallings L O Lundbeck H & Ringertz O The National Bacteriological Laboratory Stockholm SEROLOGY

The present outbreak offered an exceptional opportunity for comparing the sensitivity and specificity of certain serological tests on a well defined material. About 700 blood specimens from 276 patients and 50 healthy subjects were tested with the complement fixation reaction and the Roth microprecipitation reaction with living trichinella larvae. Skin test was performed on 142 patients and healthy subjects.

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Forty-seven cases showing an increase of the cf titer of ten or more.

... reaction. 81 and 67 per cent of the cf positive and negative cases respectively were skin test positive. There were no major differences between the groups as to the frequency of the typical symptoms or of the consumption of the sausage suspected to be the vehicle.

In the groups with negative cf reactions and moderate weak or negative precipitin reactions there was a tendency to lower frequency of positive skin tests, of the typical symptoms and of the consumption of the sausage. In the serologically negative group 37 per cent of the skin tests were positive.

Thus the *cf* reaction turned out to be specific but insensitive the precipitation reaction to be more sensitive and the weaker reactions relatively nonspecific. The skin test was sensitive but nonspecific and difficult to evaluate.

Damsgård Karen, Lofdal A. & Ringert O. The Hospital for Infectious Diseases, Karlskrona and The National Bacteriological Laboratory, Stockholm. CLINIC

Information about the clinical picture was received from 216 cases. Twenty per cent of the cases initially showed gastrointestinal symptoms. A rise in body temperature was recorded in 88 per cent of the cases, while 81 per cent had a headache, 90 per cent ache of the muscles and 89 per cent suffered from exhaustion. The characteristic oedema around the eyes and in the face was found in 72 per cent and 63 per cent had conjunctivitis. Two thirds of the cases complained of heavy perspiration. Cough was present in 27 per cent. Palpitations were found in every fifth case. Leucophilia occurred in 92 per cent of the 77 cases studied.

A group of 84 cases was followed at the clinic for infectious diseases in Karlskrona. 64 of these cases were considered unquestionable cases of trichinosis.

One third of the cases recovered within 2 weeks, while 5 of the cases still have symptoms three months after the infection.

Some complications were noted. Totally 57 cases were studied by means of the ECG and four cases of myocarditis were found. Two of these were mild while the other two still have pathological ECG after 3 months.

Pleuropneumonia occurred in four cases. No fatal cases were recorded.

TABLE
Cases with marked Eosinophilia

Case no	Age in years	Diagnosis	Measures days before op			Operation
			Curet age	portio biopsy or conisation of the cervix	Oestrogen treatment	
1	45	Uterine myoma	4	-	-	supravag amputation
2	50	Uterine myoma	4	-	-	supravag amputation
3	68	Ca of the uterine corpus	4	-	-	total extirpation
4	47	Uterine adenomyosis	II		-	total extirpation
5	47	Uterine myoma	2		-	supravag amputation
6	42	Uterine myoma	3			total extirpation
7	43	Uterine adenomyosis	6	6	-	total extirpation
8	40	Ca of the cervix st 0		6		total extirpation
9	58	Prolapse of the uterus			oestriol 1 mg X 3	plastic repair
10	III	Prolapse of the uterus	3	3	oestriol 1 mg X 2	plastic repair
11	52	Ca of the cervix st 0	6	6		total extirpation
12	45	Ca of the cervix st 0	5	5		total extirpation
13	47	Ca of the cervix st 0	7	7		total extirpation
14	33	Ca of the cervix st II	5	5		total extirpation
15	41	Ca of the cervix st 0	13	13		total extirpation

identical sites of the uterus. In the evaluation of the degree of eosinophilia at different sites, as shown in Fig. 1, at least one of the sites studied in these cases was free of tumour. In some of these cases, however, the same cancerous cancer was studied at different sites of the uterus. Hence it was not possible to compare the occurrence of

the Myometrium

Degree of eosinophilia in area				Day of cycle at op	Blood eosinophilia per mm ³	Remarks
1	2	3	4			
		++++	++	9	154	
		+	+++	30		
++++	+	+	+	9	1059	curettage 4½ months and 4 days resp before operation
++	+++		++++	9	381	allergic symptoms
	++	+++	+++	33		
+	+++	++++	+	7		
+	+		0	8	586	Widely varying no of eosinoph in corpus
++++	++++		(+)	14		
+++					768	Menopause 12 years
+++						Menopause 20 years
+	+++		(+)			Menopause 2 years
++++		-	++	21		
+-	+		0	18		
+	++		0	8		Curettage + consist of cervix 3 months biopsy of portio 2½ mths and curettage + consist of cervix 11 days before operation
			(+)	317		In addition curettage + biopsy of portio 1½ months before op

eosinophilic leukocytes in various cases by counting the absolute number in a limited area. The number was therefore estimated and the cases were classified according to a scale ranging from 0 to +++++ where 0 is 1 case.

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TABLE
Cases with marked Eosinophilia

Case no	Age in years	Diagnosis	Measures days before op			Operation
			Cu rett age	portio biopsy or conisation of the cervix	Oestrogen treatment	
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2	50	Uterine myoma	4	-	-	supravag amputation
3	68	Ca of the uterine corpus	4	-	-	total extirpation
4	47	Uterine adenomyosis	2		-	total extirpation
5	47	Uterine myoma	2		-	supravag amputation
6	42	Uterine myoma	3			total extirpation
7	43	Uterine adenomyosis	6	6	-	total extirpation
8	40	Ca of the cervix st 0		6	-	total extirpation
9	58	Prolapse of the uterus			oestriol 1 mg X 3	plastic repair
10	68	Prolapse of the uterus	3	3	oestriol 1 mg X 2	plastic repair
11	52	Ca of the cervix, st 0	6	6		total extirpation
12	45	Ca of the cervix, st 0	5	5		total extirpation
13	47	Ca of the cervix st 0	7	7		total extirpation
14	33	Ca of the cervix, st 0	5	5		total extirpation
15	41	Ca of the cervix st 0	13	13		total extirpation

identical sites of the uterus. In the evaluation of the degree of eosinophilia at different sites of the uterus sections were taken from the uterine sites shown in Fig 1. Four sections were examined of the portio (1) one from the cervix (2), at least one from the isthmus (3) and from the corpus (4) at least two sections. In these cases in which the entire uterus had been removed because of preinvasive squamous cancer of the portio (Stage 0), a large number of sections of the portio were studied.

The eosinophilic leukocytes were usually irregularly distributed in the different parts of the uterus studied. Hence it was not possible to compare the occurrence of

1c Myometrium

Degree of eosinophilia in area				Day of cycle at op	Blood eosinophilia per mm ³	Remarks
1	2	3	4			
		++++	++	9	154	
		+	+++	25	-	
+++		+	+	9	1059	curettage 4½ months and 4 days resp before operation
++	+++		+++++	9	381	Allergic symptoms
	++	+++	+++	33		
+	+++	++++	+	7		
+	+		0	8	586	Widely varying no of eosinoph in corpus
++++	++++		(+)	14		
+++					768	Menopause 12 years
+++						Menopause 20 years
+	+++		(+)			Menopause 2 years
+++++	-		++	21		
++	+		0	18		
+	+++		0	8		Curettage + conisat of cervix 3 months, biopsy of portio 2½ mths and curettage + conisat of cervix 5 days before operation
-			(+)	31*		In addition curettage + biopsy of portio 1½ months before op

eosinophilic leukocytes in various cases by counting the absolute number in a limited area. The number was therefore estimated as follows:

These cases are

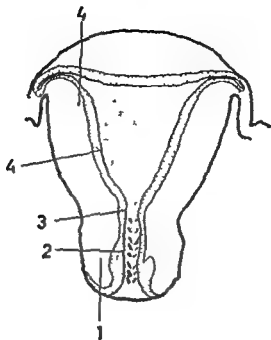


Fig 1

The figure indicates approximately the area of the uterus where eosinophilia was studied

small dose of oestrogen was administered before operation but no hormones were given in the other cases. The second group, which consisted of randomly selected cases of preinvasive cancer of the cervix comprised 22 patients who had been subjected to curettage and/or diagnostic biopsy of portio or conisation of the cervix shortly before operation. These two groups are accounted for in Tables 2 and 3.

RESULTS

As regards the slight pathological myometrical changes we considered cases in the two groups included in Tables 2 and 3 to be "normal" from a histological point of view. In these cases we found that many had few or no eosinophilic leukocytes, though other inflammatory cells such as lymphocytes, plasma cells or neutrophilic leukocytes were observed. Macrophages, occasionally containing blood pigment, were sometimes found. The localization of the eosinophilic leukocytes varied. Most frequently they were seen in the loose connective tissue both around thick and thin walled vessels. The myometrium of the corpus sometimes showed narrow bands of eosinophilic leukocytes between the muscle bundles. Here and there eosinophilic leukocytes were seen within or immediately outside blood vessels conveying the impression that they had just passed through the vessel wall. In addition the distribution might be diffuse and not related to any particular structures. The distribution of the eosinophilic leukocytes or the degree of eosinophilia showed no definite relation to the occurrence of other inflammatory cells.

TABLE 2
Cases Operated upon without Previous Curettage Biopsy of Portio or Conisation of Cervix

Case no.	Age in years	Diagnosis	Operation	Degree of eosinophilia in area				Day of cycle at op.	Menopause in years	Remarks
				1	2	3	4			
16	72	Prolapse of the uterus	Total extirpation	0	0	0	0		79	1 mg oestriol daily 3 days preoperatively
17	78	Uterine myoma	Total extirpation	+	+			11		
18	48	Uterine myoma + adenocarcinoma	Supravag amputation			0	0	23		
19	49	Uterine myoma	Supravag amp		0	0	0	12		
20	43	Dysmenorrhoea	Supravag amp		0	0	0	19		
21	42	Uterine myoma	Supravag amp		0	0	0	24		
22	45	Uterine myoma	Supravag amp		0	0	0	11		
23	43	Uterine myoma	Supravag amp		0	0	0	13		
24	41	Adenocarcinoma	Supravag amp		0	0	0	17		
25	49	Uterine myoma + Brenner tumors of ovaries	Supravag amp		0	0	0	22	-	
26	44	Uterine myoma	Supravag amp		0	0	0	9		
27	52	Uterine myoma	Supravag amp		0	0	0	15		
28	71	Prolapse of the uterus	Plastic repair	++			(+)			
29	53	Prolapse of the uterus	Plastic repair	(+)					17	Curettage at operation
30	47	Prolapse of the uterus	Plastic repair	0				16	-	
31	43	Prolapse of the uterus	Plastic repair	0				16	-	
32	47	Prolapse of the uterus	Plastic repair	0				18	-	

TABLE III
Cases Operated upon after Curettage, Biopsy of Portio or Consensus of Cervix

Case no.	Age in years	Diagnosis	Menstrual time before op.		Operation	Degree of eosinophilia in area				Day of cycle at op.	Remarks
			Curettage	Portio biopsy or consensus of the cervix		1	2	3	4		
33	33	(a of the cervix st 0)	2 days 7 weeks	2 days 7 weeks	Total extirpation	+	+	+	0	24	Parturition 4 months before operation
34	36	(a of the cervix st 0)	3 days 9 weeks	3 days 9 weeks	Total extirpation	++	+	0	0	12	
35	41	(a of the cervix st 0)	6 weeks	3 days 6 weeks	Total extirpation	++	+	-	-	21?	
36	44	(a of the cervix st 0)	3 days 7 weeks	3 days 7 weeks	Total extirpation	++	+	(+)	0	14	
37	52	(a of the cervix st 0)	3 days 7 weeks	3 days 7 weeks	Total extirpation	++	++	+	+	20	
38	46	(a of the cervix st 0)	4 days 4 weeks	4 days 4 weeks	Total extirpation	0	0	0	0	10?	
39	39	(a of the cervix st 0)	6 weeks	4 days 4 weeks	Total extirpation	++	+	-	0	12	
40	30	(a of the cervix st 0)	14 weeks	5 days 14 weeks	Total extirpation	++	+	(+)	0	17	
41	41	(a of the cervix st 0)	5 days 7 weeks	5 days 7 weeks	Total extirpation	++	+	+	+	23	Adenomyosis
42	44	(a of the cervix st 0)	5 days 7 weeks	5 days 7 weeks	Total extirpation	+	-	-	-		Extra uterine pregnancy and uterine myoma

37	Ca of the cervix at 0	5 days 6 weeks	Total extirpation	+	+	0	Menopause 6 years
44	Ca of the cervix at 0	6 days 11 weeks	Total extirpation	++	+	0	Uterine myoma
45	Ca of the cervix at 0	6 days 9 weeks	Total extirpation	+	++	(+)	16
46	Ca of the cervix at 0	7 days 4 weeks	Total extirpation	++	++	0	Menopause 2 years
47	Ca of the cervix at 0	7 days 8 weeks	Total extirpation	++	++	0	Parturition 7 months before operation
48	Ca of the cervix at 0	11 days 4 mths	Total extirpation	++	+	0	17
49	Ca of the cervix at 0	21 days 2 mths	Total extirpation	+	0	0	8
50	Ca of the cervix at 0	24 days 4 mths	Total extirpation	0	0	0	4
51	Ca of the cervix at 0	30 days 30 days	Total extirpation	0	-	0	Uterine myoma
52	Ca of the cervix at 0	2 mths 4 mths	Total extirpation	0	0	0	25
53	Ca of the cervix at 0	2½ mths 2½ mths	Total extirpation	0	0	0	-
54	Ca of the cervix at 0	1 mths 4 mths	Total extirpation	0	0	0	13
55	Ca of the cervix at 0	2½ mths 2½ mths	Total extirpation	0	0	0	Menopause 2 years
56	Ca of the cervix at 0	2 mths 4 mths	Total extirpation	0	0	0	25
57	Ca of the cervix at 0	2 mths 4 mths	Total extirpation	0	0	0	-
58	Ca of the cervix at 0	2 mths 4 mths	Total extirpation	0	0	0	13
59	Ca of the cervix at 0	2 mths 4 mths	Total extirpation	0	0	0	13
60	Ca of the cervix at 0	2 mths 4 mths	Total extirpation	0	0	0	13
61	Ca of the cervix at 0	2 mths 4 mths	Total extirpation	0	0	0	13
62	Ca of the cervix at 0	2 mths 4 mths	Total extirpation	0	0	0	13
63	Ca of the cervix at 0	2 mths 4 mths	Total extirpation	0	0	0	13
64	Ca of the cervix at 0	2 mths 4 mths	Total extirpation	0	0	0	13
65	Ca of the cervix at 0	2 mths 4 mths	Total extirpation	0	0	0	13
66	Ca of the cervix at 0	2 mths 4 mths	Total extirpation	0	0	0	13
67	Ca of the cervix at 0	2 mths 4 mths	Total extirpation	0	0	0	13
68	Ca of the cervix at 0	2 mths 4 mths	Total extirpation	0	0	0	13
69	Ca of the cervix at 0	2 mths 4 mths	Total extirpation	0	0	0	13
70	Ca of the cervix at 0	2 mths 4 mths	Total extirpation	0	0	0	13
71	Ca of the cervix at 0	2 mths 4 mths	Total extirpation	0	0	0	13
72	Ca of the cervix at 0	2 mths 4 mths	Total extirpation	0	0	0	13
73	Ca of the cervix at 0	2 mths 4 mths	Total extirpation	0	0	0	13
74	Ca of the cervix at 0	2 mths 4 mths	Total extirpation	0	0	0	13
75	Ca of the cervix at 0	2 mths 4 mths	Total extirpation	0	0	0	13
76	Ca of the cervix at 0	2 mths 4 mths	Total extirpation	0	0	0	13
77	Ca of the cervix at 0	2 mths 4 mths	Total extirpation	0	0	0	13
78	Ca of the cervix at 0	2 mths 4 mths	Total extirpation	0	0	0	13
79	Ca of the cervix at 0	2 mths 4 mths	Total extirpation	0	0	0	13
80	Ca of the cervix at 0	2 mths 4 mths	Total extirpation	0	0	0	13
81	Ca of the cervix at 0	2 mths 4 mths	Total extirpation	0	0	0	13
82	Ca of the cervix at 0	2 mths 4 mths	Total extirpation	0	0	0	13
83	Ca of the cervix at 0	2 mths 4 mths	Total extirpation	0	0	0	13
84	Ca of the cervix at 0	2 mths 4 mths	Total extirpation	0	0	0	13
85	Ca of the cervix at 0	2 mths 4 mths	Total extirpation	0	0	0	13
86	Ca of the cervix at 0	2 mths 4 mths	Total extirpation	0	0	0	13
87	Ca of the cervix at 0	2 mths 4 mths	Total extirpation	0	0	0	13
88	Ca of the cervix at 0	2 mths 4 mths	Total extirpation	0	0	0	13
89	Ca of the cervix at 0	2 mths 4 mths	Total extirpation	0	0	0	13
90	Ca of the cervix at 0	2 mths 4 mths	Total extirpation	0	0	0	13
91	Ca of the cervix at 0	2 mths 4 mths	Total extirpation	0	0	0	13
92	Ca of the cervix at 0	2 mths 4 mths	Total extirpation	0	0	0	13
93	Ca of the cervix at 0	2 mths 4 mths	Total extirpation	0	0	0	13
94	Ca of the cervix at 0	2 mths 4 mths	Total extirpation	0	0	0	13
95	Ca of the cervix at 0	2 mths 4 mths	Total extirpation	0	0	0	13
96	Ca of the cervix at 0	2 mths 4 mths	Total extirpation	0	0	0	13
97	Ca of the cervix at 0	2 mths 4 mths	Total extirpation	0	0	0	13
98	Ca of the cervix at 0	2 mths 4 mths	Total extirpation	0	0	0	13
99	Ca of the cervix at 0	2 mths 4 mths	Total extirpation	0	0	0	13
100	Ca of the cervix at 0	2 mths 4 mths	Total extirpation	0	0	0	13

TABLE III
Cases Operated upon after Curettage Biopsy or Conisation of Cervix

Case no.	Age in years	Measures taken before op.		Operation	Degree of eosinophilia in spec.				Day of cycle at op.	Remarks
		Curettage	Biopsy or conisation of the cervix		1	2	3	4		
13	31	(a of the cervix at 0	2 days 7 weeks	Total extirpation	+	+	-	0	24	Parturition 4 months before operation
14	36	(a of the cervix at 0	3 days 9 weeks	Total extirpation	++		0	0	12	
15	41	(a of the cervix at 0	6 weeks	Total extirpation	++	+		-	21?	
16	44	(a of the cervix at 0	3 days 5 weeks	Total extirpation	++		(+)	0	14	
17	52	(a of the cervix at 0	3 days	Total extirpation	++	++		+	20	
18	46	(a of the cervix at 0	4 days 4 weeks	Total extirpation	0	0		0	30?	
19	31	(a of the cervix at 0	6 weeks	Total extirpation	++	+		0	12	
20	30	(a of the cervix at 0	14 weeks	Total extirpation	++		(+)	0	17	
21	41	(a of the cervix at 0	5 days	Total extirpation	++		++	+	23	Adenomyosis
22	33	(a of the cervix at 0	3 days 3 weeks	Total extirpation	+		0	0		Extra uterine pregnancy and uterine myoma

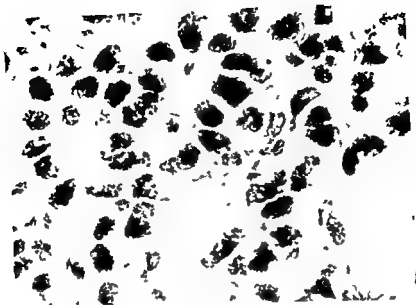


Fig 4

Case 8 Eosinophilia of the uterine portio $\times 1200$

In the group consisting of cases of eosinophilia differing considerably in severity from the reference material (Table 1) broad bands and occasionally dense carpets of eosinophilic leukocytes were seen in the same areas as in the reference material (Figs 2-4)

Also in this group other inflammatory cells might be seen, but the degree of the eosinophilia was not related here either to the inflammatory reaction

Cytologically the eosinophilic cells in the myometrium closely resembled eosinophilic leukocytes in the blood. The nucleus was frequently segmented with two or sometimes more segments (Fig 3). The granules in the cytoplasm were large, closely packed and stained intensively with eosin (Fig 4)

No clinical symptoms could be correlated to the eosinophilic myometritis

DISCUSSION

Judging from the literature, eosinophilia of the uterus of the severity described above has not been reported before. As is apparent from the reference material (Table 2), the myometrium was seldomly found to contain eosinophilic leukocytes to any measurable extent, if the operation on the uterus had not been preceded by some other surgical measure. Such intervention as biopsy or conisation of the portio or curettage shortly before the operation appeared to result in a slight increase in



Fig 2.

Case 6. Broad bands of eosinophilic leukocytes between the muscle bundles. $\times 75$

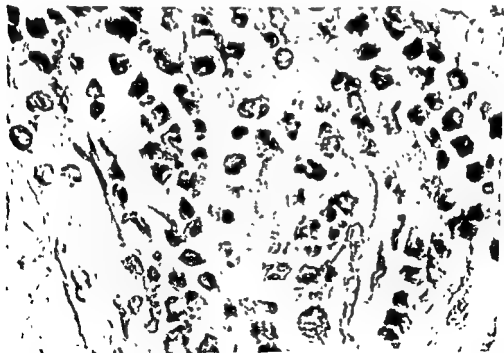


Fig 3

Case 1 Eosinophilia of the uterine isthmus $\times 760$

which conclusions can be drawn as regards a numerical variation of eosinophilic leukocytes parallel with the menstrual cycle. Nor does it permit for any evaluation of a preoperative oestrogen treatment.

No explanation can be offered for the abnormal eosinophilia observed in 15 cases (Table 1). All of the factors described above may play some role in the causation of eosinophilia. The occasionally severe eosinophilia may be simply a manifestation of a physiological variation or a tendency to an increased response. In one case an allergic condition was demonstrated and several patients presented considerable blood eosinophilia. Further investigations of uterine eosinophilia in animals and in human beings are in progress and the result will be the subject of future papers.

SUMMARY

Eosinophilic leukocytes normally occur only in small amounts in the human uterus. Surgical intervention on the uterus such as diagnostic biopsy of the cervix or portio or endometrial curettage may be followed by a moderate increase in the number of eosinophilic leukocytes. In 15 cases described here a high degree of eosinophilia in the myometrium was observed. Broad bands or dense carpets of eosinophilic leukocytes along the myometrical vessels were seen. No clinical symptoms were observed which could be related to "eosinophilic myometritis". The possible causes of this abnormal eosinophilia are discussed.

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11. *ibid.*
12. *ibid.*

the number of eosinophilic leukocytes (Table 3) This must be ascribed to the irritation of the tissue caused by the intervention, but it appears to be a fairly characteristic reaction of the myometrium Thus it is not a general consequence of tissue injury and it is rarely seen in healing surgical wounds (10) It is also evident from Table I that this eosinophilic reaction is of only short duration When the interval between the two operations was at least 3-4 weeks the number of eosinophilic leukocytes had seldomly increased

Further support for the assumption that intervention on the uterus such as biopsy or conisation results in a certain degree of eosinophilia is provided by the observation that in the biopsy material the number of eosinophilic leukocytes had seldomly increased However, the areas from which the diagnostic specimens were removed were hardly representative of those areas of the uterus where eosinophilia is most common

Rytomaa (15) showed that the number of eosinophilic leukocytes in the uterus of the rat varied with the cyclic phase In immature animals, in di-oestrus, and during pregnancy in adult animals only few or no eosinophilic leukocytes could be demonstrated The number increased rapidly in pre-oestrus and reached its maximum during oestrus A certain, simultaneous variation in blood eosinophilia could also be observed, but it was not by far as marked as in the uterus These results confirmed the observations by *Gansler* (7), who was also able to show that the administration of progesterone had the same effect as pregnancy on the number of eosinophilic granulocytes in the uterus *Gansler* (7) also found that the administration of the follicular hormone increased the number of eosinophilic leukocytes in the uterus, an observation which we have found on administration of oestriol and oestradiol (*Bjersing & Borghin*, unpublished) In guinea pigs the degree of blood eosinophilia was at the lowest during oestrus (4) and it is also known that a certain variation of the number of eosinophilic leukocytes in the blood occurs in women with normal menstrual cycles At the time of ovulation a decrease has been observed (3) *Rytomaa* (15) was unable to demonstrate simultaneous eosinophilia in any other organs

Blood eosinopenia has also been demonstrated on increased elaboration of adrenocortical steroids (16), e.g. during stress This decrease in numbers of circulating eosinophilic leukocytes is assumed to cause a certain increase in the tissue eosinophilia (10) It therefore appears reasonable to assume that any intervention causing stress, particularly in an organ as the uterus which even under normal conditions presents some degree of tissue eosinophilia, might result in increased eosinophilia The severity of the eosinophilia normally occurring after curettage or diagnostic biopsy of the portio is given in Table 3 This increased infiltration of eosinophilic leukocytes appears to persist for about 3 weeks, after which only few, if any, eosinophilic leukocytes can be demonstrated This material provides no basis on

which conclusions can be drawn as regards a numerical variation of eosinophilic leukocytes parallel with the menstrual cycle. Nor does it permit for any evaluation of a preoperative oestrogen treatment.

No explanation can be offered for the abnormal eosinophilia observed in 15 cases (Table 1). All of the factors described above may play some role in the causation of eosinophilia. The occasionally severe eosinophilia may be simply a manifestation of a physiological variation or a tendency to an increased response. In one case an allergic condition was demonstrated and several patients presented considerable blood eosinophilia. Further investigations of uterine eosinophilia in animals and in human beings are in progress and the result will be the subject of future papers.

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the number of eosinophilic leukocytes (Table 3). This must be ascribed to the irritation of the tissue caused by the intervention, but it appears to be a fairly characteristic reaction of the myometrium. Thus it is not a general consequence of tissue injury and it is rarely seen in healing surgical wounds (10). It is also evident from Table 3 that this eosinophilic reaction is of only short duration. When the interval between the two operations was at least 3-4 weeks the number of eosinophilic leukocytes had seldomly increased.

Further support for the assumption that intervention on the uterus such as biopsy or conisation results in a certain degree of eosinophilia is provided by the observation that in the biopsy material the number of eosinophilic leukocytes had seldomly increased. However, the areas from which the diagnostic specimens were removed were hardly representative of those areas of the uterus where eosinophilia is most common.

Rytomaa (15) showed that the number of eosinophilic leukocytes in the uterus of the rat varied with the cyclic phase. In immature animals, in di-oestrus, and during pregnancy in adult animals only few or no eosinophilic leukocytes could be demonstrated. The number increased rapidly in pre-oestrus and reached its maximum during oestrus. A certain, simultaneous variation in blood eosinophilia could also be observed, but it was not by far as marked as in the uterus. These results confirmed the observations by *Gansler* (7), who was also able to show that the administration of progesterone had the same effect as pregnancy on the number of eosinophilic granulocytes in the uterus. *Gansler* (7) also found that the administration of the follicular hormone increased the number of eosinophilic leukocytes in the uterus, an observation which we have found on administration of oestriol and oestradiol (*Bjersing & Borglin*, unpublished). In guinea-pigs the degree of blood eosinophilia was at the lowest during oestrus (4) and it is also known that a certain variation of the number of eosinophilic leukocytes in the blood occurs in women with normal menstrual cycles. At the time of ovulation a decrease has been observed (3). *Rytomaa* (15) was unable to demonstrate simultaneous eosinophilia in any other organs.

Blood eosinopenia has also been demonstrated on increased elaboration of adrenocortical steroids (16), e.g. during stress. This decrease in numbers of circulating eosinophilic leukocytes is assumed to cause a certain increase in the tissue eosinophilia (10). It therefore appears reasonable to assume that any intervention causing stress, particularly in an organ as the uterus which even under normal conditions presents some degree of tissue eosinophilia, might result in increased eosinophilia. The severity of the eosinophilia normally occurring after curettage or diagnostic biopsy of the portio is given in Table II. This increased infiltration of eosinophilic leukocytes appears to persist for about 3 weeks, after which only few, if any, eosinophilic leukocytes can be demonstrated. This material provides no basis on

which conclusions can be drawn as regards a numerical variation of eosinophilic leukocytes parallel with the menstrual cycle. Nor does it permit for any evaluation of a preoperative oestrogen treatment.

No explanation can be offered for the abnormal eosinophilia observed in 15 cases (Table 1). All of the factors described above may play some role in the causation of eosinophilia. The occasionally severe eosinophilia may be simply a manifestation of a physiological variation or a tendency to an increased response. In one case an allergic condition was demonstrated and several patients presented considerable blood eosinophilia. Further investigations of uterine eosinophilia in animals and in human beings are in progress and the result will be the subject of future papers.

SUMMARY

Eosinophilic leukocytes normally occur only in small amounts in the human uterus. Surgical intervention on the uterus such as diagnostic biopsy of the cervix or portio or endometrial curettage may be followed by a moderate increase in the number of eosinophilic leukocytes. In 15 cases described here a high degree of eosinophilia in the myometrium was observed. Broad bands or dense carpets of eosinophilic leukocytes along the myometrical vessels were seen. No clinical symptoms were observed which could be related to "eosinophilic myometritis". The possible causes of this abnormal eosinophilia are discussed.

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SOME ASPECTS OF THE STRUCTURE AND HISTOCHEMISTRY OF THE ADRENALS IN OBESE-HYPERGLYCEMIC MICE

By

CLAES HELLERSTROM BO HELLMAN and STIG LARSSON

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It is known that hyperadrenocorticism in mice may cause obesity and an increased number of β cells in the islets of Langerhans (*cf* Hausberger & Hausberger 1960). In studies of the islets of Langerhans in the American variety of the obese hyperglycemic syndrome in mice we also observed a considerable enlargement of the adrenals which raised the question whether an increased production of glucocorticoid steroids might be a contributory cause to the abnormal fat and carbohydrate metabolism. The biosynthesis of steroids during stimulation with ACTH was therefore studied *in vitro* (Carstensen, Hellman & Larsson 1961). Corticosterone was found to be the principal steroid formed and the total amount produced per gland was much higher in the obese hyperglycemic mice. Expressed per unit adrenal weight the amount of corticosterone formed was definitely higher in one experiment but not significantly changed in another experiment. Another metabolic characteristic of the adrenals of the obese hyperglycemic mice is the *in vitro* formation of proportionately high amounts of proline from the carbon atoms of glucose (Larsson, Hellman & Carstensen 1962).

In the present investigation the attention was directed to the structural and histochemical features of the mouse adrenals in the obese hyperglycemic syndrome and to the relation between the adrenal size and caloric intake of these animals.

MATERIAL AND METHODS

Adult mice of the strain with recessive obesity (ob/ob) were used.

From the left adrenal from 15 male obese hyperglycemic animals (40 mice) and an equal number of their lean male littermates (AN mice),

which had been allowed free access to food (caloric composition: carbohydrate 54 per cent fat 16 per cent and protein 30 per cent) was fixed in Bouin weighed on a torsion balance and after dehydration and clearing embedded in paraffin. Serial sections were cut 10 μ thick and stained with hematoxylin. Every 5th section was projected in a microscope at a magnification of 55 \times and the volume of the adrenal cortex and medulla estimated by planimetry of the image. The error of the method for both the cortical and medullary parts was calculated as less than ± 1 per cent of the mean value for the whole gland.

In another experiment the effect of a restricted food intake on the weight of the left adrenal was studied in the AO mice. The animals were allowed free access to food up to an age of about 50 days when the presence of the obese hyperglycemic syndrome could be identified with certainty. The average body weight of the 13 AO mice studied corresponded at this time to about 27 g while the body weight of the same number of controls (AN mice) was about 16 g. By then allowing the AO mice a restricted caloric intake and the AN mice free access to food the animals were of equal average body weight—about 23 g—when killed at the age of 160 days. (With free access to the same diet AO mice at this age weighed about 50 g.)

2 Qualitative studies. In addition to examination of sections stained with hematoxylin (see above) the microscopical analyses of the adrenals from the AN and AO mice were made with the following methods:

a) *Lipids.* For the study of sudanophil substance the method of Casselman (1959) with Sudan Black B dissolved in isopropanol was used.

b) *Ascorbic acid.* This substance was studied with the silver method according to Haechus (1970).

c) *Glucose 6-phosphate dehydrogenase.* The activity of this enzyme was studied in frozen sections according to the method introduced by Hess, Scarjelli & Pearse (1978) and *La arus & Braslaw* (1969) using the monotetrazolium salt MTT or the ditetrazolium salt Nitro BT as electron acceptors.

d) *Adrenaline and noradrenaline.* The chromaffin reaction as described by Hillarp & Håkfelt (1975) was applied to thin slices of the adrenals.

RESULTS

When the animals had free access to food the adrenals of the AO mice were considerably larger than those of the AN mice. The mean weights with the standard errors of the mean were for the left adrenal 3.31 ± 0.16 mg and 1.57 ± 0.04 mg respectively ($t = 10.5$, $P < 0.001$). The corresponding volume values for the material embedded in paraffin were 1.83 ± 0.10 mm³ and 0.83 ± 0.02 mm³ ($t = 10.0$, $P < 0.001$). The different adrenal sizes in AN and AO mice could be ascribed to the cortical part the volume of which was more than twice as large in the AO mice. The values obtained were for the cortex 1.63 ± 0.10 mm³ and 0.05 ± 0.02 mm³ respectively ($t = 10.1$, $P < 0.001$) and for the medulla 0.20 ± 0.01 mm³ and 0.18 ± 0.01 mm³. When plotted on double logarithmic coordinates a linear relationship between the volumes of the adrenal cortex and the body weight could be fitted for both groups taken together (see Fig. 1). The common correlation coefficient for the logarithmic values was 0.95. If the body weight of the AO mice was kept within the range of the AN mice there was no enlargement of the adrenals. For the adult AO mice given a restricted caloric intake the weights of the Bouin fixed left adrenals were 1.70 ± 0.06 mg and the corresponding values for the AN mice with free access to food 1.88 ± 0.09 mg ($t = 0.50$, $P > 0.05$).

The microscopical examination revealed that all adrenocortical layers

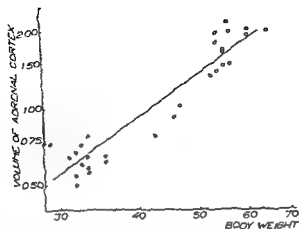
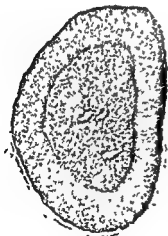


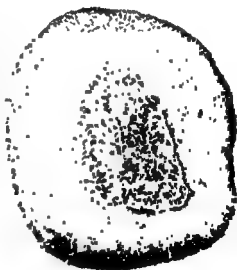
Fig 1

The relation between the volume of the adrenal cortex in mm^3 and the body weight in g for the AN mice (solid symbols, ●) and the AO mice (open symbols ○) plotted in a double logarithmic diagram. The line given in the figure represents the regression equation for the whole material.



2

Fig 2 Hematoxylin stained equatorial section of an adrenal from an AN mouse $\times 40$



3

Fig 3 Hematoxylin stained equatorial section of an adrenal from an AO mouse. In comparison with Fig 2 the cortex is considerably enlarged and the borderline between cortex and medulla diffuse $\times 40$



- Fig 4* The lipid staining with Sudan Black B of the adrenal of an AN mouse. Note the intensive black reaction throughout the cortex and the distinct borderline against the medulla $\times 50$
- Fig 5* The lipid staining with Sudan Black B of the adrenal of an AO mouse. Compared with Fig 4 the reaction is weaker and in the deeper cortical layers practically absent $\times 50$
- Fig 6* The silver reaction for ascorbic acid in the adrenal cortex of an AO mouse $\times 1100$
- Fig 7* The chromaffin reaction for adrenaline noradrenaline in the adrenal medulla of an AO-mouse $\times 1650$



8



9

10

Fig 8 The formazan staining for glucose 6 phosphate dehydrogenase in the adrenal of an A mouse. The capsule of the organ is seen in the upper right part of the figure while the white region below to the left represents the medulla $\times 160$

Fig 9 The glucose 6 phosphate dehydrogenase activity in the outer parts of the adrenal cortex in an AO mouse. Note the intensive particulate formazan staining $\times 1100$

Fig 10 The glucose 6 phosphate dehydrogenase activity in the inner parts of the adrenal cortex of an AO mouse (the medulla below to the right) $\times 1100$

were enlarged in the animals with the manifest obese hyperglycemic syndrome. The borderline between the cortex and medulla was also less distinct in the AO mice with free access to food (Figs 2 and 3). It was further apparent from the reaction with Sudan Black, that the lipid content of the adrenal cortex was considerably lower in the obese-



- fig 4 The lipid staining with Sudan Black II of the adrenal of an AN mouse. Note distinct borderline
- fig 5 in AO mouse. Compare cortical layer
- fig 6 The silver reaction for ascorbic acid in the adrenal cortex of an AO mouse $\times 1100$
- fig 7 The chromaffin reaction for adrenaline noradrenaline in the adrenal medulla of an AO mouse $\times 1650$

found to be related to the cortical part of the organ. This observation and the histochemical changes discussed above are in good agreement with the reports of an increased *in vitro* secretion of steroids from the adrenals of the AO mice (Carlsensen, Hellman & Larsson 1961) and definitely contradicts the previous statement that in these animals such adrenal changes were absent which might be associated with hyperadrenocorticism (Bleish, Vayer & Dickie 1962).

Since no changes were noted in the medullary part neither using the volumetric analyses of serial sections nor the reaction for adrenaline noradrenaline it seems relevant to concentrate the discussion of the role of the adrenals in the American variety of the obese hyperglycemic syndrome to the functional activity of the cortical part. In a summary of the available data about spontaneously occurring obesity in mice Hausberger & Hausberger (1960) concluded that the adiposity in NH mice in yellow obese mice and possibly in the New Zealand obese hyperglycemic strain was caused by some type of hyperadrenocorticism. Our observation that the size and microscopical appearance of the adrenals of the AO mice were closely related to the body weight and normalized by an elimination of their over eating may best be interpreted by regarding the increased adrenocortical activity in these animals as a secondary phenomenon. The opinion that abnormality in the adrenal function is not a primary factor in the etiology of the American variety of the obese hyperglycemic syndrome has also been expressed by Vayer (1960) who maintains that the obesity of the AO mice cannot be eliminated by adrenalectomy or reproduced by administration of corticosteroids or ACTH.

SUMMARY

The adrenal structure and histochemistry has been studied in mice with the American variety of the obese hyperglycemic syndrome. In a comparison of the obese hyperglycemic animals (AO mice) with their lean litter mates (AN mice) the following observations were made:

(1) The adrenals of the AO mice were more than twice as large as those of the AN mice. This was found to be due to an enlargement of the cortical part of the organ.

(2) While no obvious differences were noted as regards the histochemical reactions of ascorbic acid or adrenaline noradrenaline a marked depletion of lipids was noted in the adrenals of the AO mice. A high glucose 6 phosphate dehydrogenase activity was present in all cortical layers, the reaction at large appearing more intensive in the manifest obese hyperglycemic syndrome.

(3) Since the size and microscopical appearance of the

hyperglycemic animals. The depletion of lipids was especially pronounced in the inner parts of the cortex, where a positive reaction was practically absent in the AO-mice (Figs. 4 and 5). No obvious differences were noted between AO- and AN-mice with regard to the reactions for ascorbic acid or adrenaline-noradrenaline. The presence of ascorbic acid in the inner parts of the adrenal cortex of an AO-mouse is shown in Fig. 6, while the positive chromaffin reaction for adrenaline-noradrenaline in the adrenal medulla from the same animal is demonstrated in Fig. 7. When applying the reaction for glucose-6-phosphate dehydrogenase a rather intensive particulate formazan staining was found with Nitro BT as well as MTT throughout the cortex in both types of mice (Figs. 8-10). On the whole the activity of this enzyme seemed to be higher in the AO-mice with free access to food.

DISCUSSION

An increased glucose-6-phosphate dehydrogenase activity in the adrenals of the AO-mice might reflect a more intensive steroid synthesis in these animals. It has been suggested that one of the roles played by this enzyme in the adrenal cortex is to act as a generator of TPNH, which is important for hydroxylation of the steroid molecule (Haynes & Berthel 1957, Haynes 1958). The presence of lipids and ascorbic acid can be used as other criteria, which may reflect the functional activity of the adrenal cortex. The obvious depletion of lipids in the adrenals of the AO-mice is suggestive of the view of the operation of an intensive ACTH mechanism in the American variety of the obese-hyperglycemic syndrome. It may be mentioned that a diminished content of stainable lipids also has been found in the New Zealand variety of the same syndrome (Bielschowsky & Bielschowsky 1956).

An approximately equal and high glucose-6-phosphate dehydrogenase activity was observed in all layers of the mouse adrenal cortex. Cohen (1959) found that the activity of this enzyme was high in the adrenal cortex of rats except in the zona glomerulosa. Since a weaker enzyme reaction, especially in the outer part of the adrenals, might be a result of technical factors as for example thawing and refreezing processes during attachment of the organ to the cryostat chuck, care must be taken not to interpret the divergent observations regarding the glucose-6-phosphate dehydrogenase activity in the glomerulosa as necessarily reflecting a true difference between rats and mice.

Data about the adrenal weight in different types of obese mice have been given by Marshall, Andrus & Mayer (1957). While these authors found unchanged weights for artificially induced obesity (goldthio-glucose and surgically provoked hypothalamic obesity), a higher adrenal weight was found in the four adult (10-13 months old) AO mice studied. In the present investigation the existence of a pronounced enlargement of the adrenals of the AO-mice was statistically verified and

GRANULOMA GANGRENESENS WITH THE HISTOLOGICAL PICTURE OF HODGKIN'S DISEASE

By

GÖREL ÖSTBERG and OLLE ODELBERG JOHNSON

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Necrotizing, malignant nasal granuloma was first described by *Mac Bride* in England (1897), since then the disease has been referred to by a number of different names (1-7, 9-17, 19-25). In the German literature it is most commonly called granuloma gangrenescens, in the English, malignant granuloma, lethal midline granuloma, or the like. In the Scandinavian literature it has also been referred to as osteomyelitis necroticans faciei (16). The clinical picture of the disease is characteristic, while the pathological picture, the course of the disease, and the prognosis appear to be variable. Most authors have published only isolated cases, from which it is not easy to form a clear opinion on the condition. In 1958-1960 *Walton* (22, 23, 24) surveyed a large series consisting of 23 personal cases and 101 cases from the literature. All of these cases had been carefully examined, clinically and pathologically. All of the patients had been autopsied. *Walton* divided these cases of malignant granuloma, which at least initially had the same clinical picture, into three pathologically and aetiologicaly different groups. About one fourth of the patients had a malignant tumour, which *Walton* called reticuloendothelial sarcoma. In these cases the prognosis was poor, and patients survived only for a relatively short time after the onset of symptoms. In conformity with earlier descriptions of this form of malignant granuloma *Walton* used the term granuloma gangrenescens. In about 50 per cent of *Walton's* cases widespread necrotizing lesions appeared, sooner or later, in the lungs and kidneys, and these lesions, like the primary changes in the nose or oral cavity, showed the same features as *Wegener's* granulomatosis. The primary alteration thus consisted of vascular changes of a necrotizing nature. In these cases, too, the disease usually ran a fulminant course with an early, fatal issue. Cases of this type have been described in Sweden (1). The remaining 25 per cent of the patients had lesions, which were regularly situated in the nose and face, they were of the

can best be interpreted as a secondary phenomenon and not as a primary factor in the etiology of the syndrome

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GOREL ÖSTBERG and OLLE ODELBORG JOHANSON

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Necrotizing, malignant nasal granuloma was first described by *Mac Bride* in England (1897), since then the disease has been referred to by a number of different names (1-7, 9-17, 19-25). In the German literature it is most commonly called granuloma gangrenescens, in the English, malignant granuloma lethal midline granuloma, or the like. In the Scandinavian literature it has also been referred to as osteomyelitis necroticans faciei (16). The clinical picture of the disease is characteristic, while the pathological picture, the course of the disease, and the prognosis appear to be variable. Most authors have published only isolated cases from which it is not easy to form a clear opinion on the condition. In 1958-1960 Walton (22, 23, 24) surveyed a large series consisting of 23 personal cases and 101 cases from the literature. All of these cases had been carefully examined, clinically and pathologically. All of the patients had been autopsied. Walton divided these cases of malignant granuloma which at least initially had the same clinical picture into three pathologically and aetiologically different groups. About one fourth of the patients had a malignant tumour, which Walton called reticuloendothelial sarcoma. In these cases the prognosis was poor, and patients survived only for a relatively short time after the onset of symptoms. In conformity with earlier descriptions of this form of malignant granuloma Walton used the term granuloma gangrenescens. In about 50 per cent of Walton's cases widespread necrotizing lesions appeared, sooner or later, in the lungs and kidneys, and these lesions like the primary changes in the nose or oral cavity, showed the same features as Wegener's granulomatosis. The primary alteration thus consisted of vascular changes of a necrotizing nature. In these cases, too, the disease usually ran a fulminant course with an early, fatal issue. Cases of this type have been described in Sweden (1). The remaining 25 per cent of the patients had lesions, which were regularly situated in the nose and face, they were of the

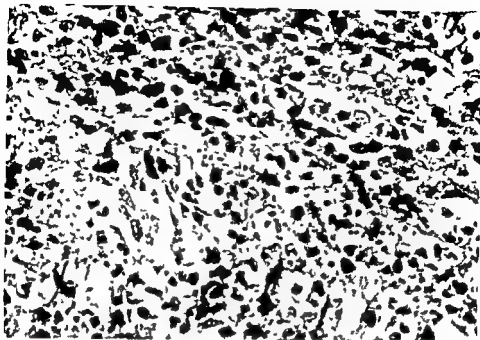


Fig 1

Biopsy specimen from nose. Granulation tissue with numerous leukocytes and lymphocytes, histiocytes and proliferating vessels. Numerous reticulum cell like elements with oval pale nuclei. Some of the cells in mitosis.
Haematoxylin eosin $\times 460$

genuine granuloma type with progressive ulcerations with abundant granulation tissue. In these cases the course was sometimes very protracted, sometimes with remissions of several years. Death was ascribed to complications of the local lesions, such as meningitis, haemorrhages or pneumonia. Walton described this group under the heading of "classical malignant granuloma".

In the below a case is described with lesions of the type which according to Walton, should be regarded as granuloma gangrenescens. Histologically, however, the changes resembled those seen in Hodgkin's disease. The disease ran a rapid, fatal course.

REPORT OF CASE

A III Para, aged 40, was operated upon by the method of Luc Caldwell in 1954 because of a perforating residual tooth root in the right maxillary sinus. In 1958 she had left sided facial erysipelas. At the end of June 1959 the left nasal cavity was obstructed with serous secretion and the anterior aspect of the left cheek as well as the upper and lower eyelids of the left eye were swelling progressively. On examination the mucosa of the left nasal cavity was swollen, slimy and red. Roentgen examination revealed no changes of the facial bones, the sinuses were of normal appearance and no lung changes were observed. Examination of a biopsy specimen (PAD 5746/59) (Fig 1) removed from the left nasal cavity showed marked inflammation with fairly widespread necrosis. The stroma showed infiltration of leukocytes, histiocytes and proliferating vessels. Almost tumour like proliferation of the inflammatory cells was observed. The pathological inflammation. The possibility of Wegener's disease of the granuloma gangrenescens



Fig 2



Fig 3

Fig 2 Untreated large ulceration over bridge of nose and severe oedema of left eyelids and cheek.

Fig 3 After treatment Nasal ulceration healed with barely discernible scar No oedema

type cannot be excluded. Wassermann's reaction in the blood was negative. In September 1959 an ulceration covered with a scab was seen in the skin on the left half of the nose and the above mentioned swelling of the soft tissues on the left half of the face had increased (Fig 2). The entire lateral aspect of the left nasal cavity was studded with ulcerations covered with scabs. In several clinical respects

1409 C. 515 1001 medication with Kenacort in doses of 4 mg three times daily Acton prolongatum was given every seventh day. After one month's treatment

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1409 C. 515 1001 patient had a catarrh of the upper airways with fever the oedema



Fig 4

Fig 4 Neck organs T 1 Larynx Bottom Centered of large lymph nodes with partly necrotic cut surface



Fig 5

Fig 5 Kidney with several pale tumour like lesions



Fig 6

Detail of Fig 5 The large tumour like lesions show a wedge shaped extension into the renal parenchyma

exposure of the bare bone. The steroid dose was increased to 30 mg of Deltison daily but without any demonstrable effect of this medication or of additional roent

progressed despite local treatment with Mycostatin and the patient sank rapidly. Death occurred in May 1960 about 11 months after the onset of the disease.

Autopsy (Ohd 494 60. Only important relevant findings are described here). **Gross findings**—The entire face was swollen particularly the left half with total closure of the left eye. In the forehead and over the left cheek firm infiltrates were seen with a smooth yellowish red surface and erosion of the skin. No external lesions were seen on the nose. The left maxillary sinus was filled with firm yellowish white tissue masses and pale yellow viscous mucus. Similar mucus was found in the left frontal sinus and ethmoidal cells. On the right side of the neck was a conglomerate of lymph nodes which were firm whitish yellow and varied in size up to the size of a chestnut (Fig 4). The axillary, inguinal and supraclavicular lymph nodes were at most the size of brown beans; they were soft and their cut surface was of normal appearance. The lungs showed signs of purulent bronchitis, multiple local pneumoniae and oedema. The cut surface of the liver showed the characteristics of congestion as well as scattered multiple pea-sized whitish yellow patches on the surface and cut surface. Similar whitish yellow patches were seen on the surface of both kidneys. A large wedge shaped almost firm whitish yellow lesion about the size of a grape was seen in the upper pole of the left kidney (Figs 5 and 6).

Microscopical findings—The lungs which were of normal gross appearance were found to contain lesions of the same type as the larger and smaller whitish yellow

nodules and closely resembled the picture seen in Hodgkin's disease. Eosinophil leukocytes were fairly rare. No reticulum fibres could be demonstrated with silver impregnation. Nowhere were any vascular changes seen.

DISCUSSION

The clinical picture was typical of the one characterizing the different forms of malignant nasal granuloma. The fulminant course and tumour like lesions seen at autopsy assign the case to the group which Walton calls granuloma gangrenescens. He described this type as reticuloendothelial sarcoma. Histologically however, the case showed in

extreme similarity from the similar one seen in Hodgkin's disease and suggested this diagnosis as more or less probable (7, 12, 20, 5, 10, 14). Walton (24) also discussed the possibility of Hodgkin's disease, which he however, categorically rejected. It is true that the clinical course with the initial necrotizing nasal changes differed considerably from that of Hodgkin's disease, but on

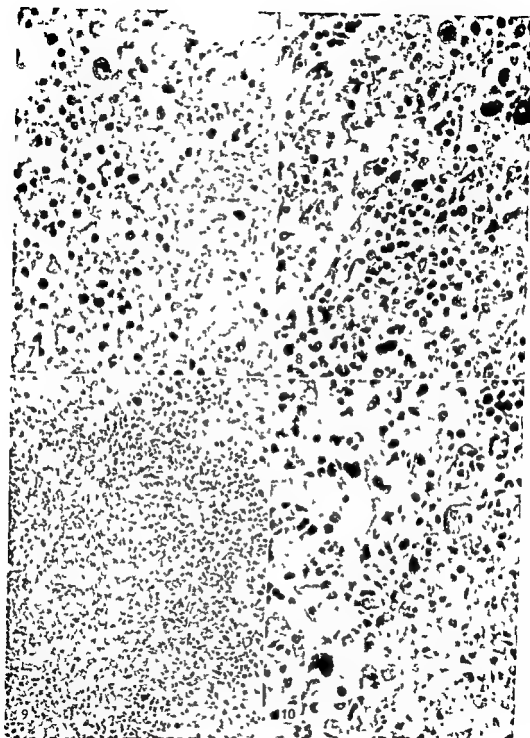


Fig 11



Fig 10

Fig

Fig

eosin X 184

Figs 7 III

- Fig 7 Autopsy specimen Oedematous necrotic subcutaneous tissues from face Leukocytes and lymphocytes as well as reticulum-cells and a few giant cells with plump irregularly shaped dark nuclei Haematoxylin eosin X 480
- Fig 8 Lymph node from mesenterium No normal structure but polymorphous tissue with different sized reticulum cells with hyperchromatic nuclei Some of these cells are in mitosis Giant cells with large nuclei

Fig 9

- Fig 10 Detail of Fig 9 Cellular polymorphism of interstitial infiltrate between liver cell trabeculae Haematoxylin eosin X 480

the other hand reticulum-cell sarcoma does not usually run such a clinical course either. It is therefore not possible to rule out the possibility of Hodgkin's disease. For diagnostic reasons the more neutral term "malignant lymphoma" would therefore appear preferable, and it would probably be more correct to regard the granuloma gangrenescens as a peculiar type of malignant lymphoma. The difficulties in differentiating the various types of this group of diseases is also apparent from the fact that a number of cases have been described in which different histological variants (reticulum-cell sarcoma, leukosis-like proliferations, plasmocytoma, Hodgkin's granuloma) have been seen in one and the same patient (8, 18). When removing tissue specimens for histological examination from necrotizing nasal lesions it would appear wise from a diagnostic and prognostic point of view to bear in mind and search for tissue changes of the above mentioned types. Cases on record as well as our case clearly show that histological recognition of the malignant, tumour-like processes in the initial nasal changes may sometimes be difficult. In order not to delay treatment of the nasal process, which may reasonably be regarded as the initial lesion, relatively large biopsy specimens should be removed at fairly short intervals. It is, however, not known with certainty whether the disease in these cases is initially local or general. Thus, in our case the affection of various organs remained concealed until autopsy. This underlines the importance of complete autopsy of all fatal cases of the disease in order to chart the spread and elucidate the nature of the pathological process. Many cases on record include no report of the findings at autopsy and are therefore difficult to evaluate. The diagnostic and therapeutic difficulties in this group of diseases may also be exemplified by another personal case. Seven years ago a tumour-like lesion covered with mucosa was seen in the nasal cavity in a 70 year old woman. Repeated biopsy regularly showed a picture resembling the one seen in Hodgkin's disease. The patient received surgical and radiological treatment. After repeated recurrences the patient, who is now 78, has been symptom-free for somewhat more than 3 years. The future will show whether the pathological process is or will in time be, local or general. Owing to the absence of nasal ulcerations this case differed from the one seen in the 40 year old woman described above.

SUMMARY

A case of granuloma gangrenescens nasi (according to Wilton's nomenclature) is described. The patient died 11 months after the appearance of the initial necrotizing nasal lesion, which at first responded favourably to roentgen irradiation and steroid therapy. Autopsy revealed generalized lesions, which histologically resembled Hodgkin's granuloma. It is stressed on the basis of literature studies that this type of granuloma gangrenescens is not so very uncommon and should be

IMMUNOCHEMICAL STUDIES ON SOME SEROLOGICAL CROSS-REACTIONS IN THE KLEBSIELLA GROUP

3. Reappearance of Fucose in the Capsular Polysaccharide of a Fucose-less Strain of K. ozanæ Type AE

By

S. D. HENRIKSEN and JORUN ERIKSEN

with the technical assistance of Unni Main

Received 14 viii 61

In a previous paper (5) it was shown that the klebsiella serotype AE, which, by definition, cross-reacts with serotypes A(1) and E(5), could be subdivided into two groups according to the chemical composition of the capsular polysaccharide. The polysaccharide of one group contained fucose, and these strains cross-reacted with types A and E in the typical manner, whereas that of the other group lacked fucose and appeared to react just like that of type E.

It was postulated that the second group was a mutant or variant of the first group and that it ought to be possible to induce it to resume fucose production. But numerous experiments with cultivation under widely varying conditions failed to bring about such a change.

It is the purpose of this paper to report the results of some more successful experiments.

MATERIAL AND METHODS

The strain 0344 of *K. ozanæ* type AE was used in the experiments. The polysaccharide of this strain (hereafter designated PS 0344 F—) had been prepared several times and had always been found to lack fucose and to react serologically like the type F polysaccharides. Polysaccharides from the fucose-containing type AF strains 0366 (designated PS 0366 F+) and S57 (designated PS S571+) and of the type E strain 026 (designated PS 026) were used for comparative studies. The serological studies were carried out with immune sera against the strains 0366 and 026.

Preparation of polysaccharides from lactose agar cultures was carried out as previously described (1, 5). Cultures in liquid media were treated as follows. The cultures were killed with phenol and the bacteria were removed by filtration through Berkefeld filters. The filtrates were evaporated in vacuum to 1/10 volume and centrifuged. The supernates were precipitated with 3 volumes of ethanol (95 per cent); the precipitates centrifuged down and washed with ethanol and redissolved in 0.5 per cent concentration in buffer solution (2 per cent CH_3COOH + 4 per cent CH_3COONa). These solutions were deproteinised by repeated shaking with chloroform and butanol, evaporated to 1/3 volume in vacuum and precipitated with 3 volumes of ethanol. The precipitates were centrifuged down, washed with ethanol, dried in vacuum, redissolved in water to a 0.2 per cent concentration and dialysed against

distilled water after which the solutions were evaporated to small volume and precipitated with ethanol. The precipitates were centrifuged down, washed with ethanol and dried in vacuum.

Hydrolysis, neutralisation and chromatography were as previously described (1, 5), likewise the quantitative precipitin determinations (4) and the gel diffusion method.

Oxidation of the polysaccharides with periodic acid was carried out as described by Staub & Tinelli (6).

EXPERIMENTAL

Experiment 1

The following fluid media were prepared:

1 Meat infusion peptone broth prepared with deionised water

2 The same medium prepared with tap water

3 A defined medium containing little nitrogen, but comparatively much glucose: $\text{Na}_2\text{HPO}_4 - 1$, $\text{KH}_2\text{PO}_4 - 0.3$, $(\text{NH}_4)_2\text{SO}_4 - 0.03$, $\text{K}_2\text{SO}_4 - 0.1$, $\text{NaCl} - 0.1$, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O} - 0.02$, $\text{CaCl}_2 \cdot 6\text{H}_2\text{O} - 0.002$, $\text{FeSO}_4 - 0.0001$ and glucose - 1 per cent. According to Wilkinson *et al.* (7) such a medium should give a good yield of polysaccharide.

4 The same medium as in 3, but with 1 galactose instead of glucose.

Two liter portions of these media were divided into 200 ml portions in Erlenmeyer flasks. The cultures were inoculated with strains 0344 and incubated at 37° C for 48 hours, killed with phenol and treated as described above.

The results showed that the polysaccharide prepared from medium 2 contained a trace of fucose, but none of the others.

Experiment 2

Two 10 liter portions of medium 2 were divided into 0.5 l portions in 1 l Erlenmeyer flasks. Both sets of flasks were inoculated from the same broth culture of strain 0344 and incubated for 24 hours at 37° C,

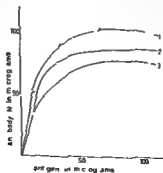


Fig. 1

Quantitative precipitin analyses in immune serum against strain 026 type F with type AF and type F capsular antigens.

- Curve 1 Antigen PS 026 (○) and antigen PS 344 F— (×)
 Curve 2 Antigen PS 0344 F+
 Curve 3 Antigen PS 0366 F+

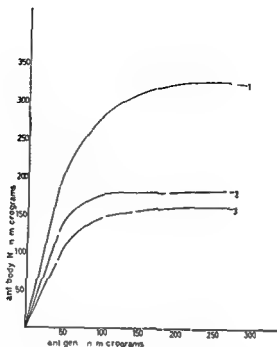


Fig 2

Quantitative precipitin analyses in immune serum against strain 0366 fucose containing type AF with type AF capsular antigens

Curve 1 Antigen PS-0366 F+
 Curve 2 Antigen PS-0344, F-
 Curve 3 Antigen PS-0344 F+

one set of flasks being left standing quietly, the second set being shaken on a shaking machine during incubation to improve aeration

Polysaccharide prepared from the stationary cultures showed a weak, but definite fucose spot on chromatography of hydrolysate, whereas polysaccharide from the shaken cultures, which grew much more heavily, showed a larger fucose spot. The latter polysaccharide (PS-0344, F+) was used for quantitative precipitin analyses in comparison with the polysaccharides mentioned before.

The results shown in Table 1 and Fig 1 appeared to be as expected, with PS-0344, F+ in an intermediate position between PS-0344, F- and PS-026 on one side, and PS-0366, F+ on the other. This is as one might expect if the strain had partially regained its capacity of fucose production.

The results shown in Table 2 and Fig 2, on the other hand, did not support this simple explanation, as PS-0344, F+ showed a weaker instead of a stronger cross-reaction in the immune serum anti-0366, F+ than PS-0344, F-. This suggested that fucose in PS-0344, F+ might have a different location in the polysaccharide molecule than in PS-0366, F+, since it seemed to block or eliminate some of the antigenic determinants responsible for the cross-reactivity in anti-366 serum instead of creating new, or better fitting, cross-reactive groupings.

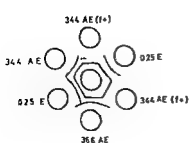


Fig 3 a

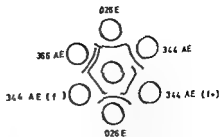


Fig 3 b

Fig 3 a In the central basin immune serum against strain 0366 type AE

Fig 3 b In the central basin immune serum against strain 026 type E

An additional experiment gave some support to this view. The polysaccharides PS 557 F+ and PS-0344 F+ were subjected to oxidation with periodic acid (6). After hydrolysis and chromatography of the oxidised polysaccharides the former still showed an apparently unchanged fucose spot whereas in the latter not a trace of fucose remained suggesting that the fucose in this polysaccharide is bound in some other manner than in PS 557 F+. The results of gel precipitation tests are shown in Figs 3 a and b and seem to agree with the other results without adding any new information.

DISCUSSION

This study was carried out in order to try to induce a fucose less strain of type AE believed to be a mutant or variant of a fucose containing strain to resume fucose production and to revert to the specificity typical of type AF.

The results show that this strain was indeed capable of fucose production although it is uncertain whether this production was induced by the experimental conditions. But contrary to expectations the specificity of the strain did not seem to have reverted to that characteristic of other fucose containing strains but appeared to be still farther removed from the typical type AE than the fucose less type E. This suggested that the fucose could probably not occupy the same positions in the polysaccharide molecules in the two cases. The results of oxidation with periodic acid lend some support to this view since in one case fucose appeared to be largely unaffected by the oxidation whereas in the second case it seemed to have been completely eliminated.

Thus although the results confirmed our suspicion that the fucose less strains of type AE should be able to produce fucose and likewise confirms that the serological specificity is changed by the presence of fucose the rest of our hypothesis remains unconfirmed as it is still uncertain whether a fucose less strain can be made to return to the original serological specificity. Although this was somewhat disap-

pointing, it is interesting that a sugar component of a *Klebsiella* capsular polysaccharide apparently can change positions in the molecule, thereby causing changes of the antigenic specificity. The fact that the presence and location of fucose only causes moderate changes of the specificity, may indicate that only comparatively few determinant structures may be involved.

If similar changes are common in the *Klebsiella* group—and certain previous observations (2, 3) might be taken to indicate that this could be the case—it might explain some of the variability of the specificity of *Klebsiella* capsular polysaccharides, as well as some irregular results of serological studies. It is an important question whether such changes could be induced by changes in the growth conditions. If it could be shown that certain conditions of growth favoured specific changes, this might help to explain why certain serotypes by preference occur in certain pathological conditions, such as ozaena.

SUMMARY

Fucose was found in the capsular polysaccharide of a previously fucose-less *Klebsiella* strain, originally classified as type AL. The appearance of fucose reduced rather than augmented the cross reactivity of the strain with other fucose-containing strains of type AL. This and the fact that fucose in this polysaccharide disappeared after oxidation with periodic acid in contrast to the fucose of other strains, suggest that the fucose may have a different location in this strain than in the others.

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IMMUNOCHEMICAL STUDIES ON SOME SEROLOGICAL CROSS-REACTIONS IN THE KLEBSIELLA GROUP

*6. Chemical Analysis of the Capsular Polysaccharide
of Klebsiella Type 3 (C) and of two Cross-Reacting Strains*

By

JORUN ERIKSEN and S. D. HENRIKSEN
with the technical assistance of Unni Mann

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Klebsiella type 3 is one of the very rare examples of a capsular type which is shared by several different species. The sero-type 3 has so far

organisms are identical

The purpose of this and the following paper is to report some immunochemical studies of several different strains of this type as well as of two strains which were found to cross-react strongly with type 3 immune sera without being able to absorb all the antibody

MATERIAL AND METHODS

Strains *K. rhinoscleromatis* 9204, an old laboratory strain *K. ozaenae* 3828 isolated here from a case of ozaena, *K. pneumoniae* F10\Y, one of Julianelle's strains *Aerobacter (Klebsiella) aerogenes*, MA73, a strain isolated from urine. These 4 strains appeared to be true type 3 strains. B1076/48, a strain isolated from urine and 349, a strain isolated from a sample of water, cross reacted strongly in type 3 sera but failed to absorb all antibody.

Polysaccharides were prepared from lactose agar cultures by the cold water extraction method described previously (3). The crude polysaccharides were deproteinised by shaking with chloroform butanol, dialysed and reprecipitated with ethanol.

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pointing, it is interesting that a sugar component of a *klebsiella* capsular polysaccharide apparently can change positions in the molecule, thereby causing changes of the antigenic specificity. The fact that the presence and location of fucose only causes moderate changes of the specificity, may indicate that only comparatively few determinant structures may be involved.

If similar changes are common in the *Klebsiella* group—and certain previous observations (2, 3) might be taken to indicate that this could be the case—it might explain some of the variability of the specificity of *klebsiella* capsular polysaccharides, as well as some irregular results of serological studies. It is an important question whether such changes could be induced by changes in the growth conditions. If it could be shown that certain conditions of growth favoured specific changes, this might help to explain why certain serotypes by preference occur in certain pathological conditions, such as ozaena.

SUMMARY

Fucose was found in the capsular polysaccharide of a previously fucose-less *klebsiella* strain, originally classified as type A12. The appearance of fucose reduced rather than augmented the cross-reactivity of the strain with other fucose-containing strains of type A12. This and the fact that fucose in this polysaccharide disappeared after oxidation with periodic acid in contrast to the fucose of other strains, suggest that the fucose may have a different location in this strain than in the others.

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IMMUNOCHEMICAL STUDIES
ON SOME SEROLOGICAL CROSS REACTIONS
IN THE KLEBSIELLA GROUP

■ *Chemical Analysis of the Capsular Polysaccharide
of Klebsiella Type 3 (C) and of two Cross Reacting Strains*

By

JORUN ERIKSEN and S. D. HENRIKSEN

with the technical assistance of Unni Mairi

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Klebsiella type 3 is one of the very rare examples of a capsular type which is shared by several different species. The sero type 3 has so far been found in *K. rhinoscleromatis*, *K. ozaenae*, *K. pneumoniae* and *Aerobacter (Klebsiella) aerogenes*. Qualitative serological tests and absorption tests have indicated that the capsular polysaccharides of these organisms are identical.

The purpose of this and the following paper is to report some immunochemical studies of several different strains of this type as well as of two strains which were found to cross react strongly with type 3 immune sera without being able to absorb all the antibody.

MATERIAL AND METHODS

Strains *K. rhinoscleromatis* 9204 an old laboratory strain *K. ozaenae* 3828 isolated here from a case of ozaena. *K. pneumoniae* 1741
streptococcus
48
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ser

Polysaccharides were prepared from lactose agar cultures by the cold water extraction method described previously (3). The crude polysaccharides were deproteinised by shaking with chloroform butanol dialysed and reprecipitated with ethanol.

Qualitative analysis of the polysaccharides was carried out after hydrolysis with 3N H₂SO₄ neutralisation with CaCO₃ and determination

as follows:
1. Reducing sugar: Fehling's solution (10% CuSO₄ and 10% NaOH) and 40% oxalate for reducing sugar and uronic acid. 2. Amino sugar: 1% ninhydrin for amino sugar. 3. Carbohydrate: 1% water (10% 40% oxalate for reducing sugar and uronic acid. 4. Nitrogen: 1% naphthoresorcinol and CCl₃COOH for non-reducing sugar and ninhydrin for amino sugar.

Quantitative determination of the sugar components was carried out with Somogyi's copper reagent (7) according to Flood Hirst & Jones (2). Uronic acid was determined according to Johansson, Lindberg & Theander (5). Ash was determined by incineration in platinum crucibles and nitrogen by a micro kjeldahl procedure.

RESULTS

Some of the reactions which distinguish between the strains are shown in Table 1. It is seen that several different biochemical types are represented: *K. rhinoscleromatis* (92/04), *K. ozaenae* (3828—as far as we know the first ozaena strain of this sero-type to be detected outside Indonesia (8)), one of the classical Friedlander strains of *Julianella* (6), F 10 N 1, with a reaction pattern similar to that of *Citrobacter*, *K. aerogenes* (or *K. pneumoniae* according to Kauffmann) (M473), an indol-positive strain of the same biotype (B 1076/48), and a strain which might be classified as a member of the genus *Enterobacter* of *Hormaeche & Edwards* (4), 349.

TABLE 2
Composition of Capsular Polysaccharides of the Strains

Strain	Percentage of Components						
	galactose	glucose	mannose	rhamnose	uronic acid	N	Ash
<i>K. pneumoniae</i> strain F 10 N 1	15	0	22	0	26	0.7	8.5
<i>K. rhinoscleromatis</i> strain 92/04	13	0	20	0	23	0.8	7.9
<i>K. ozaenae</i> strain 3828	15	0	24	0	26	0.2	9.1
<i>K. aerogenes</i> strain M473	17	0	22	0	24	0.6	9.7
<i>K. aerogenes</i> strain B1076/48	13	0	20	0	27	0.5	8.9
' <i>Enterobacter</i> ' strain 349	12	10	13	7	23	1.4	7.8

The results of qualitative and quantitative analyses of the polysaccharides are shown in Table 2. It is seen that 5 of the polysaccharides are indistinguishable from one another by these analytical data, whereas one of the cross-reacting strains (349) contains glucose and rhamnose in addition to the three sugars contained in the other polysaccharides. Also the mannose and possibly the galactose content of this polysaccharide was lower. The N-content, which was higher in this polysaccharide, could not be brought further down by shaking with butanol-chloroform. The uronic acid contained in these polysaccharides could not be identified by the methods used.

SUMMARY

Four different strains of *Klebsiella* sero-type 3, classified as the species *rhinoscleromatis*, *ozaenae*, *pneumoniae* (*Citrobacter*?) and *aerogenes* had capsular polysaccharides of the same qualitative and quantitative composition, likewise a cross-reacting strain of *K. aerogenes* whereas a second strongly cross-reacting strain related to *Enterobacter* contained two additional sugar components.

TABLE 1
Some Cultural and Biochemical Characteristics of the Strains

Strain	Cultures on lactose agar	Lactose	Glucose	Saccharose	I	M	N	C	Lr	Motility
92/04	Large flat slimy, confluent blue	A ₁	A	A ₂	-	+	-	-	-	-
3829	Large flat slimy confluent blue turning yellow	A ₁	A	A ₂	-	+	+	+	W +	-
110 N	Domed discrete moderately mucoid yellow	A ₁	A + G	A	-	+	-	+	+	-
M 173	Large raised confluent very slimy, yellow	A ₁	A + G	A	-	-	+	+	+	-
11076/48	Domed discrete moderately mucoid yellow	A ₂	A + G	A	+	+	+	+	+	-
349	Flat transparent non mucoid yellow, later slimy and blue	A ₁	A + G	A	-	-	+	+	-	+

A Acid G Gas Figures indicate the day when the reaction became positive in the case of delayed reactions

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IMMUNOCHEMICAL STUDIES ON SOME SEROLOGICAL CROSS REACTIONS IN THE KLEBSIELLA GROUP

7 Serological Reactions of some Strains of Type 3(C) and some Cross-Reacting Strains

By

S. D. HENRIKSEN and JORUN FRIKSEN
with the technical assistance of Linn Marie

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MATERIAL AND METHODS

The strains were the same as in the preceding paper (1). The serological methods were the same as previously described (2, 3).

RESULTS

Table 1 shows the results of the capsular reaction. Of the 4 strains believed to be true type 3 strains 3 gave comparable titers in all immune sera whereas the fourth (3828) gave 2 to 3 fold lower titers than the homologous strain in all sera. Of the two cross reacting strains one 349 gave equally high titers in all sera as the type 3 strains, whereas the second B1076/48 gave 16 to 32 fold lower titers in all sera except the homologous one.

TABLE 1
Capsular Reactions of the Strains in Different Immune Sera

Strains	Immune Sera				
	Anti 110	Anti M473	Anti 9204	Anti 349	Anti B1076
F10 N 1	2048	256	512	512	128
9204	1024	256	512	128	256
3828	256	128	128	128	64
M473	1024	256	512	512	256
B1076/48	64	8	32	32	128
349	2048	256	256	512	256

Titers are given as reciprocal of serum dilution

Fig. 1 shows the results of quantitative precipitation determinations in the immune serum against the Friedländer strain F10 N 1. The homologous antigen brings down a good deal more antibody than any of the heterologous antigens, whereas the antigens from the 3 other type 3

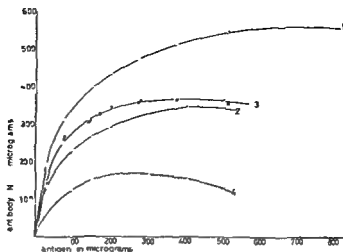


Fig 1

Quantitative precipitation determinations in immune serum against *Klebsiella pneumoniae* strain F10. The curves show μg antibody \searrow per 0.5 ml serum

Curve 1 Antigen F10 \searrow

Curve 2 Antigen 349

Curve 3 Antigens 92/04 () 3828 (x x x) and MA73 (o o o o)

Curve 4 Antigen B1076/48

strains bring out exactly the same quantity of antibody. The cross-reacting antigen prepared from strain 349 brings out nearly, but not quite, as much antibody, and the antigen prepared from strain B1076/48 precipitated only about half as much.

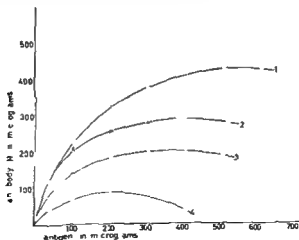


Fig 2

Quantitative precipitation determinations in immune serum against *Aerobacter aerogenes* MA73. The curves show μg antibody \searrow per 0.5 ml serum

Curve 1 Antigen MA73

Curve 2 Antigens F10 \searrow () 92/94 (x x x) and 3828 (o o o)

Curve 3 Antigen 349

Curve 4 Antigen B1076/48

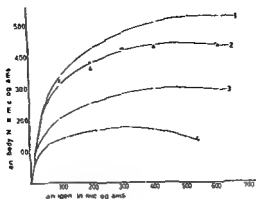


Fig 3

Quantitative precipitation determinations in immune serum against H1 rhinoscleromatis 92/04. Curves show μg antibody $\%$ per 0.5 ml serum

Curve 1 Antigen 92/04

Curve 2 Antigens F10 \ \ () 3828 (X X X) and M473 (O O O)

Curve 3 Antigen 349

Curve 4 Antigen B1076/48

Figs 2 and 3, presenting the results in the two other type 3 immune sera (anti M473 and anti 92/04), reveal practically the same situation. The homologous antigen removes more antibody than any of the heterologous type 3 antigens. The latter antigens give analytical data which fall nicely on the same curve. The curves representing the antigen from strain 349 fall only slightly to moderately lower, whereas the antigen prepared from strain B1076/48 precipitates only a minor fraction of the antibody.

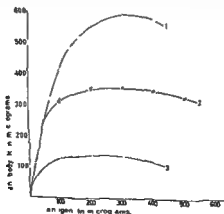


Fig 4

Quantitative precipitation determinations in immune serum against Enterobacter strain 349. Curves show μg antibody $\%$ per 0.5 ml serum

Curve 1 Antigen 349

Curve 2 Antigens F10 \ \ () 92/04 (X X X) M473 (O O O) and 3828 (□ □ □)

Curve 3 Antigen B1076/48

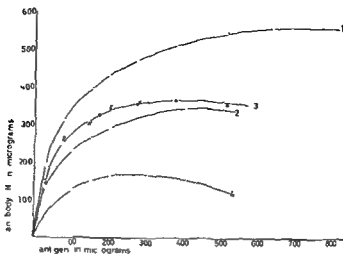


Fig 1

Quantitative precipitation determinations in immune serum against *Klebsiella pneumoniae* strain F10 N 1. The curves show μg antibody N per 0.5 ml serum

Curve 1 Antigen F10 N 1

Curve 2 Antigen 349

Curve 3 Antigens 92/94 () 3828 (X X X) and MA73 (O O O)

Curve 4 Antigen B1076/48

strains bring out exactly the same quantity of antibody. The cross reacting antigen prepared from strain 349 brings out nearly, but not quite, as much antibody, and the antigen prepared from strain B1076/48 precipitated only about half as much.

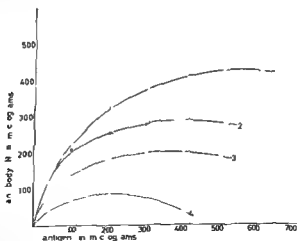


Fig 2

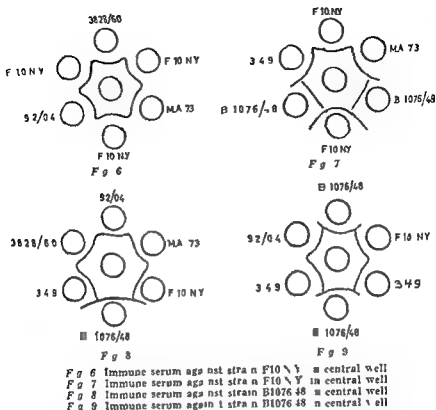
Quantitative precipitation determinations in immune serum against *Aerobacter aerogenes* MA73. The curves show μg antibody N per 0.5 ml serum

Curve 1 Antigen MA73

Curve 2 Antigens F10 N 1 () 92/94 (X X X) and 3828 (O O O)

Curve 3 Antigen 349

Curve 4 Antigen B1076/48



of the homologous antigen in Figs 1 to 3. In all of the figures where antigen from strain B 1076/48 is represented the partial cross reactivity of this antigen is revealed by spur formation or in one case (Fig 11) by the absence of a visible precipitate.

DISCUSSION

The results confirm that the serological specificity of the type 3 capsular polysaccharide is the same regardless of the biochemical reaction pattern of the organism. In addition they show that sera against these organisms contain antibodies reacting specifically with the homologous organism only. Which type of antigen is involved remains unknown but it is reasonable to assume that it may be the O antigen which according to Kauffmann and his associates (4, 5, 6, 7) is responsible for the O group of *Klebsiella* strains. This being the case the results indicate that the O groups of our 4 strains are different. This is consistent with the early results obtained by Dutch workers (8, 9, 10) and also seems compatible with the results obtained by Kauffmann and his collaborators (4, 7). That these strain specific antigens must be present in small quantities only is suggested both by the precipitin curves

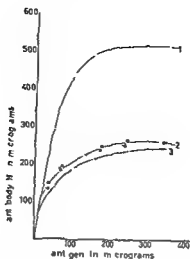


Fig 5

Quantitative precipitin determinations in immune serum against *Aerobacter aerogenes* B1076/48. Curves show μ g antibody N per 0.5 ml serum

Curve 1 Antigen B1076/48

Curve 2 Antigens F10 Δ γ (), 92/04 (x x x), M473 (o o o)
and 3828 (\square \square \square)

Curve 3 Antigen 349

Fig 4 shows the results obtained with immune serum against strain 349. In this case the homologous antigen precipitates a good deal more antibody than any other antigen, all the 4 true type 3 antigens precipitate exactly the same quantities of antibody, and the antigen from strain B1076/48 removes less than 25 per cent of the antibody.

Fig 5 shows the results obtained with the immune serum against strain B1076/48. The homologous antigen removes about twice as much antibody as any heterologous antigen. The 4 type 3 antigens again remove exactly equal quantities of antibody, and the antigen from strain 349 removes only very slightly less.

Figs 6 to 12 show the results of gel precipitation tests. These are in excellent agreement with those just described. In all figures it is apparent that the 4 type 3 antigens share an important antigen, believed to be the capsular antigen. The polysaccharide of strain 349 contains an antigen which by this method, appears identical with the type 3 antigen. Apparently in this study the quantitative precipitin method has a slightly better power of resolution than the gel diffusion method. We gained the same impression in other studies of this series. This method may not be quite as suitable to such antigen-antibody systems as the present as *e.g.* to protein-antiprotein systems.

In Figs 6, 7, 10, 11 and 12 it is apparent that the homologous antigen produced a second indistinct precipitate band, closer to the antigen basin. This seems to be an antigen which is present in lower concentration or it diffuses more slowly than the capsular antigen, or both. This antigen may be the one which is responsible for the higher curves

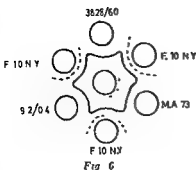


Fig 6

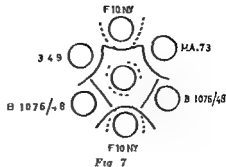


Fig 7

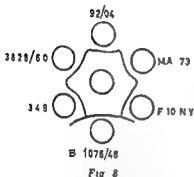


Fig 8

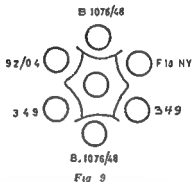


Fig 9

Fig 6 Immune serum against strain F10 NY in central well

Fig 7 Immune serum against strain F10 NY in central well

Fig 8 Immune serum against strain B1076/48 in central well

Fig 9 Immune serum against strain B1076 48 in central well

of the homologous antigen in Figs 1 to 3. In all of the figures where antigen from strain B 1076/48 is represented, the partial cross-reactivity of this antigen is revealed by spur formation or, in one case (Fig 11), by the absence of a visible precipitate.

DISCUSSION

The results confirm that the serological specificity of the type 3 capsular polysaccharide is the same regardless of the biochemical reaction pattern of the organism. In addition they show that sera against these organisms contain antibodies reacting specifically with the homologous organism only. Which type of antigen is involved remains unknown, but it is reasonable to assume that it may be the O-antigen which, according to Kauffmann and his associates (4, 5, 6, 7), is responsible for the O group of *Klebsiella* strains. This being the case the results indicate that the O-groups of our 4 strains are different. This is consistent with the early results obtained by Dutch workers (8, 9, 10), and also seems compatible with the results obtained by Kauffmann and his collaborators (4-7). That these strain-specific antigens must be present in small quantities only, is suggested both by the precipitin curves,

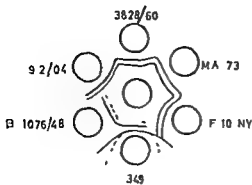


Fig 10

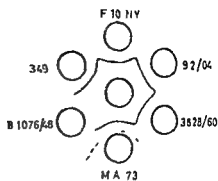


Fig 11

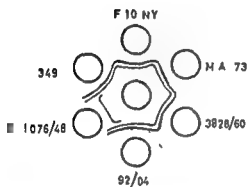


Fig 12

Fig 10 Immune serum against strain 349 in central well

Fig 11 Immune serum against strain MA73 in central well

Fig 12 Immune serum against strain 92/04 in central well

showing that much antigen is needed before the top of the homologous curve is reached, and also by the gel precipitation results. They may perhaps be present in too small quantities to have an influence on the data obtained by chemical analysis.

It is interesting that in this case, where the capsular antigen is the same, this antigen does not prevent the detection of the second antigen. It seems practicable to determine antibodies against these second antigens by means of precipitation tests, provided that the capsular antigen either was identical, as in this case, or entirely different with no cross-reaction. This is a possibility which easily might be tested.

One of the consequences of these results is that in similar work the possibility should always be kept in mind that the assumed capsular polysaccharides may be contaminated with other antigens, which are not easily eliminated.

The results obtained with the two cross reacting strains are of some interest, since they show that the strain B 1076/48 which appeared to have the same composition as the type 3 antigens, only shows a moderate cross-reaction with type 3, whereas the antigen from strain 349 reacts with very nearly all of the type-specific antibody contained in type 3 immune sera, in spite of the fact that it contains two sugar components which are absent from type 3 polysaccharide, and also differs in the contained quantity of maltose and perhaps of galactose. It seems

quite remarkable that a strain which differs from type 3 to such a high degree could have this almost identical serological specificity. However, the explanation may be that this polysaccharide actually was a mixture of two substances, only one of which will cross-react with type 3. In this connection it may be worth mentioning that all of the capsular reactions carried out with this strain showed that only a minority of the organisms actually gave a capsular reaction, whereas the majority appeared to be acapsular. Also young cultures of the strain were non-nucoid, and only became very slimy after two or three days. Therefore, the 'capsular' polysaccharide of this strain might contain more of the O antigen than the other polysaccharides. This problem might be solved if it were discovered whether rhamnose were detectable in the specific precipitates formed by this polysaccharide and a true type 3 immune serum.

SUMMARY

Capsular polysaccharides from strains of *Klebsiella rhinoscleromatis*, *K. ozaenae*, *K. pneumoniae* and *Aerobacter (Klebsiella) aerogenes*, all classified as type 3, had the same serological specificity. They contained a small quantity of a second, strain-specific antigen, possibly an O antigen.

Of two cross reacting strains one had almost the same specificity as type 3, but contained two additional sugar components, whereas the other, which gave only a moderate cross-reaction with type 3, had the same composition as the type 3 polysaccharides. The implications of these findings are discussed.

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TWO NEW SALMONELLA SPECIES
SALMONELLA HISINGEN = 48:a:1,5,7 AND
SALMONELLA LUNDBY = (9), 46:b:e,n,x

By

B BACKELIN, V. HJILBORN and L. RUTQVIST

Received 2 VIII 61

Two new *Salmonella* species were isolated from fish-flour imported from Angola

Salmonella hisingen The biochemical pattern of the strain is as follows. It does not ferment inositol, rhamnose, lactose, sucrose, and salicin, nor does it liquefy gelatin or decompose urea. It ferments rapidly arabinose, dulcitol, glucose (with gas), maltose, mannitol (with gas), xylose, and after 2 days, sorbitol. It gives positive reaction in Stern's glycerol fuchsin broth after 6 days, and negative in d,l-1,2-tartrate, citrate after 14 days. Mucate gives positive reaction after 8 days and sodium-malonate after 2 days. The H₂S and methyl red reactions are positive, the Voges-Proskauer reaction negative. Thus with the exception of the malonate reaction the strain belongs to *sub-genus I*.

The antigenic structure is 48 a 1,5,7

Salmonella lundby The biochemical pattern of the strain is as follows. It does not ferment inositol, lactose, sucrose, and salicin. It liquefies gelatin (Kohn + Lautrop) after 3-4 days and ferrochloride-gelatin after 6 weeks. It does not decompose urea, and ferments rapidly arabinose, dulcitol, glucose (with gas), maltose, mannitol (with gas), xylose, rhamnose, and sorbitol. It gives positive reaction in Stern's glycerol fuchsin broth. D-tartrate is late and irregularly positive or negative, l- and i-tartrate negative after 14 days, citrate positive after 2 days, mucate and malonate positive after 1 day. The H₂S and methyl red reactions are positive, the Voges-Proskauer reaction negative. The strain belongs to *sub-genus II*.

The antigenic structure is (9), 46 b e,n,x

The strains isolated have been sent to the International Salmonella Centre in Copenhagen and the proposed names *S. hisingen* and *S. lundby* have been accepted.

We are greatly indebted to professor F. Kauffmann for his valuable help

SUMMARY

Two new *Salmonella* species *S. husingen* — 48 a 157 and *S. lundby* — (9) 46 b e n x are described. The strains were isolated from fish flour imported from Angola.

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AN UNUSUAL PARACOLOBACTRUM AEROGENOIDES AS THE CAUSE OF NOSOCOMIAL URINARY TRACT INFECTION

By

ARVE LASTAD

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During the daily routine work in our laboratory, we became aware of a new type of paracolon organism which had previously been unknown to us. This organism suddenly occurred as a frequent cause of urinary tract infections, especially in patients with urological diseases requiring surgical intervention.

Our attention was first drawn to this organism by the characteristic colour and the sharp smell of bromthymolblue lactose agar cultures of these organisms, as described later.

Secondly our attention was drawn to this organism by its high frequency in urinary tract infections in the surgical departments. This made us suspect a nosocomial outbreak of urinary tract infections.

The purpose of this work was to examine the morphological, biochemical and serological properties, as well as the antibiotic sensitivity of the strains, and to study the epidemiology of the outbreak. The investigations were performed on strains of this paracolon organism isolated from cases of urinary tract infections from the last months of 1958 through 1960.

MATERIAL

During the period from October 1958 to January 1961 51 strains of paracolon organisms with unusual properties were collected from the urine of 45 patients. 41 were under treatment in Rikshospitalet Oslo and 2 were outpatients but one of the patients had undergone urological treatment in the surgical department A 5 months before we first found the paracolon organism in her urine.

One additional strain (SA1136) was isolated from the pleural exudate of a patient under treatment in surgical department A.

37 patients were male and 8 were female. 25 patients were hospitalized in surgical department A (SA), 6 patients in surgical department B (SB), 12 in other departments of Rikshospitalet and 2 were outpatients (PR2 and PR3). Of the 14 patients who had been treated elsewhere than in the surgical departments, 6 had been admitted to or had undergone urological examination in one of the surgical departments at some previous time in 3 cases (MB2, PR2, N1) the clinical records were unavailable and in the remaining 5 cases there was no evidence that the patients had been treated or examined in the surgical departments but 2 of the latter patients had symptoms of urinary tract infections (MB3, MB4) and 2 had been catheterized weekly for a long time before the microbes were isolated (N3, R2).

Our strains were collected in the course of the daily routine. All the strains which showed the characteristic behaviour and reactions described later, were lyophilized for later examination. We believe that the majority of strains occurring during the period from October 1958 to January 1961, are included in our material.

Table 1 shows the distribution of the patients in different diagnostic categories.

TABLE 1

Diagnosis	No. of patients
Hyperplasia prostatae	7
Hydronephrosis	1
Pyelonephritis chronica	4
Cancer prostatae	3
"	3
"	3
"	3
"	1
Tuberculosis of the prostate	5 (total)
"	3
"	1
Stricture of the urethra	1
Stricture of the urethra, fistula	1
Total	45

METHODS

Motility was examined by inoculating the strains in U formed tubes containing a semisolid agar. The tubes were examined after 2 to 3 days at 37° C. Two strains were investigated after flagellar staining (Jensen 1959). They were found to have peri-

) Formation of acid
carbohydrates and

1) The production of indole in a casein broth (Kauffmann 1954) 2) Gelatin liquefaction

Immune sera were prepared separately against formalin killed H antigens and heated O antigens of the four strains SB1, M41, C49 and C510.

Our epidemiological studies were carried out as follows: From the patients' clinical records, we noted the kinds and dates of investigations and surgical treatments on the urinary tract, recently and in the past, previous bacteriological findings and the time of the first isolation of the organism in the urine. The intervals between urological investigation or surgery and the finding of this organism were noted.

With 10 strains we made the following studies (Henriksen 1950): 1) Cultivation on lactose bromthymolblue agar plates at 37° C, with transfers of the yellow colonies to new plates every 24 hours. 2) Fermentation tests in 1 per cent lactose peptone water with Andrade's indicator with transfers to new tubes after showing acid reactions. Serial subcultures in the same medium during 3-4 days. 3) Cultures in the fluid medium which showed an acid reaction, were also spread onto lactose bromthymolblue agar plates.

RESULTS

All our strains were Gram negative rods of variable lengths but mostly short rods. The growth was very good on agar, blood agar and lactose agar. The colonies were non-haemolytic. They looked delicate and transparent. After a growth of 24 hours on bromthymolblue lactose agar which was green before inoculation, the colonies and agar plates became yellow in that part of the plate where the growth was confluent, and deep blue in that part of the plate where the colonies grew singly. There was a very sharp separation between the yellow and blue zone. From the yellow part, we observed a very pungent, unusual, and very characteristic smell. The colonies do not smell like cultures of *E. coli* or *Aerobacter* on lactose agar. After 48 hours' growth, the lactose was fermented over the whole agar plate and the smell was more intense than the day before.

Most of the strains grew as S-forms (41 strains), but a few produced mucoid growth (M-forms, 10 strains). The smell of the M-forms was not so sharp as the smell of the S-forms. The mucoid property was so pronounced that slimy threads could be drawn from the colonies by means of the wire loop. After a few hours on the desk at room temperature, the slimy and moist colonies became flattened and dry. To keep them as true M-forms, we had to place the cultures in humid and cold rooms.

The organisms were motile. After growth in swarm agar in U-tubes, the organisms were highly motile after 1 to 3 days' growth, M-forms possibly less motile than S-forms. M-forms often needed one day more to migrate from one end of the U-tube to the other.

The following biochemical reactions were uniformly positive: Formation of acid from mannitol, maltose, glucose, sucrose and cellobiose, formation of gas from glucose, formation of acetyl-methylcarbinol, growth on citrate, reduction of nitrates and growth in KCN-medium. One strain, SA24, failed to ferment maltose and did not grow in KCN-medium.

These reactions were uniformly negative: Formation of indole, methyl-red reaction, formation of urease and desamination of phenylalanine. Formation of acid from lactose did not occur within 24 hours in any of the strains. In 6 strains formation of lactose did not occur

within 21 days, while in the other strains formation of lactose occurred after 2-11 days. Gelatin was not liquefied by any strain and the production of hydrogen sulphide was very slight (17 strains) or none (34 strains).

We have also examined the resistance of the strains to sulphonamides, penicillin, streptomycin, chloramphenicol, tetracyclins, erythromycin, oleandomycin, nitrofurantoin and kanamycin. All strains were sensitive to chloramphenicol and moderately sensitive to nitrofurantoin. About one half of the strains were moderately sensitive, the others resistant to oxytetracyclin, and all the strains were resistant to all the other substances.

Our investigations of amino acid decarboxylase in 29 strains of this organism showed a weak or delayed arginine decarboxylase activity but neither glutamic acid nor lysine decarboxylase. The ornithine decarboxylase seemed to be strong.

The results of the preliminary agglutination and absorption tests suggested that the strains could be divided into 4 serological groups: group a consisting of 19 strains from 19 patients, group b of 7 strains from 7 patients and group c of 17 strains from 15 patients, as well as a fourth group d, of 8 unclassifiable strains from 8 patients. A study of the morphology, biochemical reactions and antibiograms of our strains, indicated close relationship between the strains. Furthermore a study of the clinical records of the patients, suggested that these strains belonged to the same nosocomial outbreak. Finally, in some instances different isolates from the same patients fell into different serological groups. All this suggested that the preliminary results might be misleading, and it therefore became necessary to carry out a more exact serological analysis with separate H- and O antigens and corresponding immune sera. The results of these studies (Table 2) seemed to confirm that the strains had the same H- and O antigens, with the possible exception of a few strains, which gave some irregular results. It is possible that some of these irregularities, which mainly occurred with strains in M form, may have been due to the presence of an M antigen.

Figure 1 illustrates the cumulation of strains from patients in different clinical departments and at different time.

Table 3 illustrates the correlation in time between urological examinations or surgery and our bacteriological findings.

Table 4 illustrates the operations which have been done to these patients before this unusual organism was isolated from the urine.

In 28 patients there is a correspondence in time between urological examinations or surgery and detection of this organism in the urine. In 3 patients we cannot find any correlation. In 5 cases

TABLE 2
*Preliminary Serological Studies with Sera Produced with Formalin Killed Organisms
 and Studies with H and O Sera Prepared According to Kauffmann*

Strain	Mor- pho- logy	Sera produced with formalinkilled organisms					H and O sera prepared according to Kauffmann						
		Anti MA1	Anti MA1 abs SBI	Anti MA1 abs SA2	Anti SBI	Anti SBI at's MA1	Anti MA1 H	Anti SA12 H	Anti SA18 H	Anti SBI O	Anti MA1 O	Anti SA2 O	Anti SA18 O
a	SA 7	640	0	0	1280	0	1280	1280	640	80	320	640	0
	SA 23	1280	0	0	1280	0	1280	1280	1280	160	320	640	0
	SA 26	640	0	0	1280	0	1280	1280	1280	40	640	640	0
	SB 1	320	0	0	2560	0	1280	1280	1280	160	320	320	0
	N 2	640	0	0	1280	0	1280	1280	1280	80	640	640	0
b	SA 22	2560	1280	320	2560	0	1280	1280	1280	160	640—	640	0
	SB 2	1280	1280	320	640	0	1280	1280	1280	160	640	640	0
	MA 1	2560	1280	160	640	0	1280	640	320	160	1280	1280	0
c	SA 2	40960	>5120	80	0	0	1280	1280	160	160	640	1280	0
	SA 27	>5120	>5120	160	20	0	1280	1280	80	320	640	1280	0
	SA 20	>5120	>5120	320	0	0	1280	1280	40	160	320+	640	20
	SA 12	>5120	>5120	320	0	0	1280	1280	160	160	320+	640—	0
	VA 3	20480	>5120	320	0	0	1280	1280	320	160	640—	640+	0
	VB 2	>2560	>2560	160	40	0	1280	1280	320	320	640—	640	0
	SA 18	0	0	0	0	0	1280	160—	640	0	40+	0	320
d	SA 9	0	0	0	0	0	1280	0	0	40	320+	640	0
	SB 5	0	0	0	0	0	1280	640	640	0	40	160	320
	N 1	0	0	0	0	—	1280	1280	640	0	320	160	160

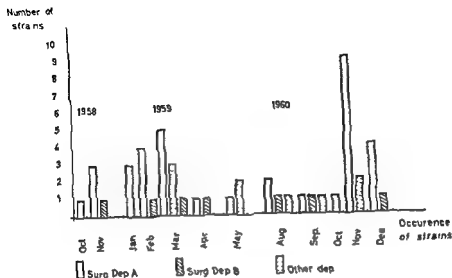


Fig. 1

Frequency of the occurrence of strains in different departments

The studies of the breakdown of lactose in 10 strains showed the following results: 1) After cultivation on bromthymolblue lactose agar plates with transfers of the yellow colonies to new plates every 24 hours for 4 days, the colonies retained their colour and smell. 2) After transferring organisms from lactose peptone water tubes, where the indicator showed acid reaction (after 2-7 days' growth), the indicator showed acid reaction in the subcultures within 24 hours, likewise in 3-4 consecutive subcultures. 3) After spreading onto bromthymolblue lactose agar plates of all of the cultures in fluid medium which showed acid reaction, the majority of the colonies on the plates still kept their unusual properties described before, while others were different after 24 hours' growth. In some colonies of the usual appearance, we observed sectors of a different yellow colour. The sectors were of variable size. They were light yellow when the rest of the colony was blue, or sharp yellow to orange when the rest of the colony was of a light yellow colour. By

TABLE 3

Interval between Catheterization, Urological Examination or Urological Surgery and Detection of Paracolobactrum aerogenoides Strains

Time	4-10 days	2 weeks-1 month	> 1 month-2 years	Unknown or uncertain*	Records unavailable
No. of patients	14 (35%)	9 (22.5%)	5 (12.5%)	11 (30%)	5

*Includes patients who had been treated in other hospitals before and patients from whom *P. aerogenoides* had been isolated in the urine sample taken during the first cystoscopy in our hospital (5 cases or 22.5%).

TABLE 4

The Distribution of the Different Forms of Urological Investigations or Urological Surgery, which Have Been Made before Isolating P. aerogenoides

Operation	Number of patients	% of all of the urological operations
Cystoscopy	25	62.5%
Catheterization	4	10.0%
Transurethral resections	4	10.0%
Urethra pyeloplastics	3	7.5%
Other urological surgery	4	10.0%
Unavailable records	5	
	45	

subcultivating the sectors, colonies obtained after 24 hours' growth on bromthymolblue agar plates failed to show the properties described before. The entire colony had a yellow-green colour, and the surrounding medium was fermented and the colonies were yellow. From these cultures we have not observed the smell described before.

On the plates, subcultured from tubes with acid reaction, three types of colonies were found. The usual one with characteristic smell and colour, the colonies with sectors just described and a third type with yellow-green colour and without the characteristic smell described before. These colonies were just like those subcultivated from the sectors. These colonies showed acid reaction in lactose peptone water tubes within 24 hours. They have the same biochemical properties as the original colonies except the lactose fermentation and urease reaction. The formation of acid from lactose and formation of urease, came within 24 hours. After culturing the new type of yellow-green colonies on agar plates without lactose for 24 hours, subcultures were made on agar plates daily for 7 days. From the second, fourth and sixth subculture, subcultures were made on bromthymolblue lactose agar plates. The colonies still kept their properties. Colonies of the original type with the characteristic smell and aspect, were not observed.

Acid and gas formation from lactose occur by culturing both kinds of colonies in lactose peptone water tubes sealed with paraffin.

Before discussing our results, it may be mentioned that a strain of the same kind of organism was isolated from a pleural exudate in August 1960. The patient had been in the Surg. Dep. A for about 2 weeks when the strain was isolated. The patient had got chok thorax after a penetrating lesion. Samples of exudate examined on the 5th and 8th of August were negative, but on the 16th we isolated this organism. After a period of chloramphenicol therapy, the organism was not found on the 8th and 17th of September. Morphologically the strain was an S-form. It belonged to the first postulated serological group a. The biochemical properties and the sensitivity to antibiotics of this strain, were as reported.

DISCUSSION

The properties of the organism described above indicate that it belongs to the family *Enterobacteriaceae* (Bergey 1957). The IMViC reactions, the fermentation of cellobiose (Koser 1926) and the KCN test indicate relationship to *Aerobacter* rather than to *Escherichia* but the usual *Aerobacter* has a different smell when growing on lactose agar and ferments lactose within 24 hours. Borman, Stuart & Wheeler 1944 suggested the establishment of a genus *Paracolobactrum* to include the lactose negative coliform organisms and proposed the following three species: *P. aerogenoides* for those related to *Aerobacter* genus, *P. intermedium* for those resembling the intermediate coliform organisms and *Paracolobactrum coliforme* for those related to *Escherichia*. The organisms isolated in our laboratory and described here seem to belong to the species *Paracolobactrum aerogenoides* of the family *Enterobacteriaceae*.

The data given in Figure 1 show that urinary tract infections caused by *P. aerogenoides* were frequent in a period from October 1958 to April 1959 and in a period from August to December 1960 in the surgical departments of Rikshospitalet, Oslo. In the first half of 1961 these infections still continued to occur.

The characteristic smell and colour of cultures on bromothymolblue lactose agar were very unusual. The smell probably is due to some volatile breakdown product of lactose when the organism grows on lactose agar plates. The smell increases with the number of yellow colonies on the plate. The characteristic deep blue colour after 24 hours' growth of those parts of the plates where colonies are few and well separated suggest that this lactose breakdown process may be weak or slow. Heavy confluent growth is necessary to produce an acid reaction within 24 hours. When lactose is fermented in peptone water tubes this characteristic smell is not produced and in this medium acid production starts much later and may not even be apparent within 21 days. This shows that the acid production on lactose agar plates depends upon the presence of atmospheric oxygen.

This suggests that the decomposition of lactose which occurs on plates is not the usual fermentation but represents an entirely different process where the decomposition takes an unusual pathway, probably an oxidative process requiring molecular oxygen. It seems that the enzymes responsible for the usual type of lactose fermentation may be absent or defective causing lactose breakdown to follow a different pathway. Whether it is the permease or the betagalactosidase or some other enzyme which determines the nature of the process on plates and the nature

The most unusual feature of our organism may be the fact that it has developed an alternative pathway of lactose decomposition in contrast to the usual paracolon organisms.

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agglutination test with an immune serum against an *E. coli* M strain indicated that the M antigen of our mucoid strains was the same as that of *E. coli*.

Our results seem to indicate that all our strains may have the same origin, but that there are certain antigenic variations.

We are unable to find any report in the literature about *Paracolo bacterium aerogenoides* as the cause of nosocomial urinary tract infections. Nosocomial *Klebsiella* infections were reported by Ørskov (1952), and from Norway (1953) at the same time, and *Proteus* infections by Story (1954). Juel, Kvittingen & Sveeggen (1955) examined 150 cultures isolated from urine, during a period with accumulated hospital infections. Dutton & Ralston (1957) reported a material from a urological ward. Omland (1960) examined 33 strains of *Proteus rettgeri* isolated from the urine of 22 patients under treatment in the surgical departments of Rikshospitalet, Oslo, from 1958 to 1959. He regarded a nosocomial mode of transmission of *Proteus* probable. Omland concludes that 'even if the facts brought out in this study do not allow any conclusions on the mode of transmission, urological instruments such as cystoscopes and resectoscopes are suspected to play a part'.

Almost all of our patients has undergone more or less extensive urological examinations or surgery including cystoscopy, catheterization, transurethral resections or, in a few cases, urethteroplastics (Table 4). The essential information given in Table 3, is that there seems to be a correlation in time between urology and bacteriological findings. Isolation of *P. aerogenoides* almost only from surgical departments in patients without such bacteriological findings before, seems to justify the assumption that almost all of the urinary tract infections caused by this microbe must have a nosocomial genesis. The organisms, serologically related, are found in different patients coming from different parts of the country. This also seems to justify the assumption of a nosocomial genesis of these urinary tract infections. Figure 1 shows that the microbe is very frequently represented in samples from Surg. Dep. A and the microbes show a periodical appearance.

Our material does not allow for any conclusions to be drawn as to the transmission of infections within the surgical departments. Almost all of the urological examinations represented in our material, are carried out in small preoperative or dressing rooms, usually not in the operation theaters nor in the wards. The majority of urological examinations before the isolation of this organism were made with cystoscopes (Table 4). It is reasonable to suspect cystoscopes, resectoscopes and catheters of being the cause of transmission of infections in urological wards. Retrospectively it is very difficult to prove this theory. A completely effective sterilization procedure is very difficult to apply to such instruments. We cannot show that patients have been transferred from one surgical department to another. Only one patient (No. 2), who was under treatment in the Neurosurgical department, was sub-

The fact that the strains keep their properties after several subcultures on lactose agar plates, indicates that this organism may be a mutant rather than an example of adaptive enzyme formation. The yellow-green colonies found together with the original colonies and the colonies with sectors in the peptone water tubes with acid reaction may be mutants responsible for the acid reaction within 24 hours. In tubes without acid reaction, we found only the original colony type. By culturing the yellow-green colonies in peptone water tubes, an acid reaction develops within 24 hours.

The sectors described and the fact that we are unable to turn this yellow-green colony back to the original colony type by changing the medium, indicate that these organisms also are mutants rather than the result of adaptive enzyme formation (Hahn & Wissemann 1951).

It will lead too far in this paper to pursue the studies of lactose metabolism in this unusual *P. aerogenoides* any further. We can now only point out that from a biochemical point of view, this organism seems to be very interesting and unusual.

The serological studies of these organisms show some very interesting results. The first agglutinations with formalin-killed organisms as antigens for serum production and agglutination, made us postulate three to four different serological groups. In the literature from the last 15 years, we find a few reports on the serology of *P. aerogenoides*. Eveland & Faber jr (1953) made an antigenic study of 58 cultures of *P. aerogenoides*, belonging to one definite biochemical group, 32011 of Stuart *et al* (1943). They found a heterogeneous serology with the presence of a number of antigenic groups. Christensen (1947) found that thirteen out of sixteen cultures of *P. aerogenoides* (isolated from stools) were serologically related.

Our investigations show that the strains are serologically related with respect to H- and O-antigens. The difference in the serology in our first studies, can be explained by M-antigens or very strongly developed H-antigens masking the O-agglutination. Especially in the group c strains, the strong H-antigenic reaction may have masked the O agglutination of serum anti SB1. Group d seems to be a heterogeneous serological group, with some serological variations. Some of the strains seem to be like those in the other groups with respect to the H- and O antigens, while others are different. This may be caused by an M-antigen. The majority of M strains are found in this group d.

There does not seem to be any important serological difference between our strains apart from the fact that some strains have an M-antigen in addition to the H- and O antigens and that the H-antigen appears to have been lacking in occasional cultures (serum anti SA18-H). It seems possible that the M-antigen may have been the cause of some of the irregular results of the agglutination tests. Previously (Henriksen 1949, 1954) it has been found that strongly mucoid strains of all genera of the tribus *Escherichieae* may produce the same M-antigen. Slide

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Later Tchen & Vennesland (1955) found such an enzyme in wheat germ Walker (1957) and Saltman, Kuntake, Spolter & Stitts (1956) described a similar enzyme in extracts from crassulacean plants, while Rosenberg, Capindale & Whalley (1958) found the same kind of activity in chloroplast extracts from spinach leaf. The enzyme is also present in the chemoautotrophic bacterium *Thiobacillus thiooxidans* (Suzuki & Werkman 1957).

More recently Stu, Wood & Stjernholm (1961) have described an enzyme from some extracts of *Propionibacterium shermanii* which catalyses a third, reversible reaction



MATERIALS AND METHODS

Preparation of cell free extracts The meningococcal strains were the same which have previously been used, and were maintained in the same way (Jysum, Borchgrevink & Jysum 1961). The cells were harvested from the surface of solid media as described. After washing with saline the bacteria were suspended in the buffer to be used for the actual experiment, and the suspension was treated at maximum oscillation for 2 minutes in a NSF ultrasonic disintegrator (60 watts). The cell debris was removed by centrifugation at 20 000 g for 30 minutes, and the supernatant was the source of the enzymes. All preparative manipulations were carried out at ice water temperature.

Assay Malic dehydrogenase activity was followed with a Hilger and Watts Uvispec spectrophotometer by measuring the change in absorbancy at 340 m μ associated with the oxidation of DPNH. The reactions were performed in quartz cells with a light path of 1 cm.

Exchange reactions with the analysis of an incorporation of C¹⁴ labelled CO₂ into a pool of metabolites

The procedures of Utter were performed with two sidearms. In reactions where incorporation of C¹⁴ was measured by direct counting on filter paper disks. In the second sample the entire supernatant from the reaction vessel after centrifugation was gassed with tank CO₂ to remove bubbling.

The hydrazones were extracted from the carbonate solution. The solution and the 2,4-dinitrophenylhydrazones were extracted with ethylacetate and the ethylacetate gently evaporated. In order to separate the mixture the 2,4-dinitrophenylhydrazones were transferred to the origin of Whatman No. 4 chromatographic paper. The chromatograms were developed with 3 per cent NH₄OH. The sheets were stripped from the

... and evaporation. The methods used

PHOSPHOENOLPYRUVIC CARBOXYLASE ACTIVITY IN EXTRACTS FROM NEISSERIA MENINGITIDIS

By

K. JYSSUM and SINDEL JYSSUM

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Recent studies in this laboratory have shown that cell free extracts from *Neisseria meningitidis* contain a pyruvic oxidation system and a lactic dehydrogenase dependent on DPN (Jyssum 1960). A phosphoenolpyruvic transphosphorylase which functions with ADP is also present (Jyssum, Borchgrevink & Jyssum 1961). Meningococci furthermore contain the enzymes considered necessary for the conventional tricarboxylic acid cycle with the exception of a malic dehydrogenase (Jyssum 1960). Malic acid however is linked with pyruvic acid by a DPN dependent malic enzyme. Since the condensing enzyme is readily supplied with Acetyl coA by the pyruvic oxidation system a crucial point for the function of a cyclic reaction through the tricarboxylic and dicarboxylic acids apparently must be the synthesis of oxalacetic acid. The present paper deals with this problem.

Three different reactions have been described which result in the synthesis of oxalacetic acid by carboxylation of a C₃ unit. Uller & Wood in 1946 first showed that crude pigeon liver extracts would in the presence of NTP fix C¹⁴O into the β-carboxyl carbon of oxalacetate. On purification the enzyme was found to require ITP and not ATP as cofactor in addition to Mn. Uller & Kurahashi (1953) accordingly described the following reversible reaction catalysed by a phosphoenolpyruvic carboxylase:



The obligatory nature of ITP does not extend to plants since a corresponding enzyme which is widely distributed in plant tissues requires ATP (Mazelis & Vennesland 1957). The corresponding enzyme from yeast also specifically requires ATP (Cannata & Sjöquist 1959).

Bandurski & Gruner (1953) purified a phosphoenolpyruvic carboxylase from spinach which catalysed an irreversible formation of oxalacetate



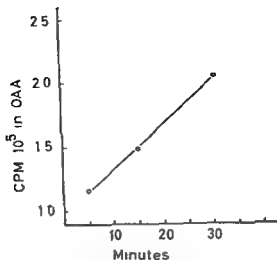


Fig 1

Fixation of $C^{14}O_2$ by the synthesis of OAA from PEP in the presence of cell free extracts from *N. meningitidis*

The composition of the reaction mixture was 7.5 μ moles tris as buffer pH 7.4, 3 μ moles $MgCl_2$, 10 μ moles PEP, 4 μ moles $NaH^{14}CO_3$ with an activity of 10^7 CPM under the conditions of the assay and 0.5 ml cell free extract dilution. The data are given as CPM in the isolated OAA (cf. methods).

presence of Mg^{2+} ions (Speck 1949), some of this pyruvate probably is the result of a decarboxylation of the oxalacetate synthesized. The complete absence of radioactivity in pyruvic acid in the present experiments may suggest that the labelling of the oxalacetate is restricted to the β carboxyl group (Walker 1957).

The uptake of radiocarbon into oxalacetic acid proceeded approximately linearly with time in the way it has been recorded in Figure 1. In contrast to this the faint activity of the α oxoglutarate spot was highest after 5 minutes, and became steadily weaker after 15 and 30 minutes.

The synthetic of oxalacetic acid was also investigated in a quantitative assay by means of malic dehydrogenase. In these experiments a mixture with 2.5 μ moles $MgCl_2$, 6 μ moles PEP, 7.5 μ moles tris as buffer pH 7.4, 2 μ moles phosphate pH 7.4 and 0.5 ml meningococcus cell free extract dilution was incubated at $24^\circ C$ for various periods of time. The experiment was terminated by the addition of 2 ml 5 per cent perchloric acid. After centrifugation the supernatant was treated with 1.15 ml of 1.1 M K_2HPO_4 . The precipitate was removed by centrifugation and the supernatant which had pH 6.2 was analysed with malic dehydrogenase and DPNH. At this hydrogen ion concentration this enzyme is not inhibited (Tchen & Vennesland 1955). In such an experiment 0.72 μ moles oxalacetic acid was found after 15 minutes, and 1.22 μ moles after 20 minutes incubation.

for measurements of radioactivity will be described elsewhere. The activity distribution in paper chromatograms was recorded by means of paper strip recording in a thin window gas flow counter. The activity of other material was measured in a window-less gas-flow counter. The basic procedures were otherwise similar to those previously used (Jyssum, Borchgrevink & Jyssum 1961).

Chemicals. The barium-silver salt of phosphoenolpyruvic acid (PEP), oxalacetic

NaHCO_3 was purchased from The Radiochemical Centre, Amersham, Bucks. England. The malic dehydrogenase was the product of Boehringer & Soehne GmbH, Mannheim, Germany.

RESULTS

Synthesis of Oxalacetic Acid

At first a reaction corresponding to equation (1) was considered. The experimental approach was based on the incorporation of C^{14}O_2 into a pool of oxalacetic acid in the way it has been described under methods. Experiments were run with ATP, ITP, ADP and IDP as cofactors. In other successive experiments MnCl_2 , GSH and the nucleotide coenzymes were omitted. In some experiments the radioactivity of C^{14} in the NaHCO_3 in each reaction vessel was increased to a total of 50 μc , while the ordinary procedure calls for only 0.9 μc (Colowick & Kaplan 1955). In such experiments no incorporation of labelled CO_2 into the oxalacetate pool occurred.

Since thus no reversible reaction appeared to exist in which C^{14}O_2 could be incorporated into oxalacetic acid, attention was turned to the direct analysis of other possible mechanisms which might function. A system was arranged for an analysis of a reaction such as suggested by equation (2).

A solution containing 3 μmoles MgCl_2 , 10 μmoles PEP, 75 μmoles tris as buffer pH 7.4 and 0.5 ml meningococcus cell free extract dilution was allowed to run for various periods of time at 24° C. The reaction was stopped, and the ketoacids extracted and separated according to the procedures described. Chromatography showed well defined spots which corresponded to the 2,4-dinitrophenylhydrazones of oxalacetic acid. The quantity of oxalacetic acid increased approximately linearly with time when analysed after 5, 15 and 30 minutes. The chromatograms also showed faint spots corresponding to the pyruvic acid hydrazones. A very weak spot with the R_f value of α -oxoglutaric acid hydrazone was also found.

When $\text{NaHC}^{14}\text{O}_3$ with a total activity of 3.5–10 CPM under the conditions of radioassay employed, was included in the reaction mixture and chromatograms were produced in the way it has been described, the oxalacetic hydrazones were found to be highly radioactive. Scanning of the chromatograms showed that the weak spot of the α -oxoglutaric acid hydrazone was also very faintly labelled. No activity was detected in the pyruvic spots. Since the oxalacetate is decarboxylated in the

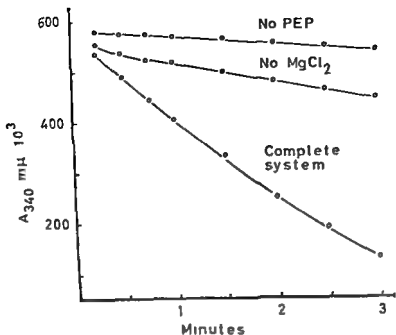


Fig. 3

Requirements for substrate and cations in the PEP carboxylase reaction

In one extract dilution which was frozen and kept at such a temperature overnight the loss of activity was 62.8 per cent. It is a consequence of these findings that only fresh cell free extracts, maintained at ice water temperature were used during the present experimental investigations.

The Cofactor Requirement

The requirements for substrate and coenzymes appear from some data compiled in Figure 3 and Figure 4. From the curves presented a requirement for magnesium may be seen.

The rate of oxidation of DPNH in the combined spectrophotometric assay with various cations was further studied in a series of analyses such as shown in Table 1. These experiments revealed Mn^{++} as the most effective activator while Co^{++} was the second best. The activating effect of Mg^{++} on the other hand is significantly less than that of the two former. In remarkable contrast to the effect of these cations is the failure of Cd^{++} , Ca^{++} and Zn^{++} to activate the PEP carboxylase.

In the table is also included some data illustrating the effect of various cations when they are present in the system in addition to Mg^{++} . These data show that not only are Cd^{++} , Ca^{++} and Zn^{++} ineffective as

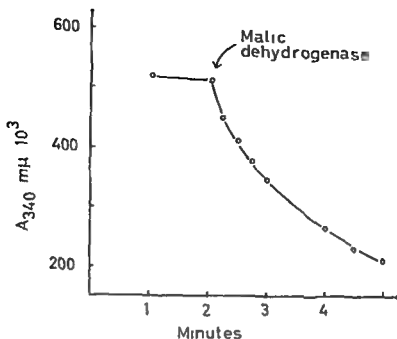


Fig. 2

Oxidation of DPNH by PEP in the presence of malic dehydrogenase. The reaction mixture contained in a volume of 2.0 ml: 75 μ moles tris as buffer pH 7.4, 2.5 μ moles MgCl_2 , 3 μ moles KCN , 0.15 μ moles DPNH, 2 μ moles PEP and 0.5 ml meningo-coccus cell free extract dilution. After 2 minutes 50 μ l malic dehydrogenase dilution with 2.5 μ g enzyme protein was added.

Oxidation of DPNH by PEP in the Presence of Malic Dehydrogenase

Cell free extracts from *Neisseria meningitidis* have been found to be free from malic dehydrogenase activity (Jyssum 1960). In order to be able to measure the carboxylation from PEP to OAA in a direct spectrophotometric technique by way of a linked reaction, malic dehydrogenase had to be added. The effect of such an addition has been illustrated by the data recorded in Figure 2. When sufficient malic dehydrogenase is added in order to make the carboxylase the rate limiting factor, this assay may serve as a method for quantitative studies of the carboxylase activity.

Since with crude meningo-coccal extracts the DPNH oxidation is pronounced, cyanide had to be added in the way it has previously been described (Jyssum 1960).

An orientation concerning the stability of the crude enzyme showed a significant fall of activity during storage at room temperature. At 20° C the activity in one extract dilution fell from 188 when the activity is given as $\Delta A_{340} \cdot 10^3$ in the first 60 seconds, to 160 within a period of two hours. During this interval the decrease in activity was approximately linear. When the enzyme was kept at ice water temperature, however, no significant loss of activity occurred during four hours of observation. In extracts which were frozen and kept at 20° below zero a loss of activity was also found, particularly in more diluted samples.

an oxidation of DPNH. This discrepancy was not caused by the presence of cyanide in the present system. A more stable DPNH, however, may be the result of a higher pH during the present experiments (pH 7.4) than that used by the authors mentioned (pH 7.0).

DPNH was on the other hand rapidly oxidized by Cu^{++} . These ions also completely inhibited the PLP carboxylase activity. Fe^{++} which disturbed the photometric assay by the oxidation of Fe^{++} to Fe^{+++} also appeared to inhibit the carboxylase completely.

The Effect of Inhibitors

The effect on the reaction of some usual SH reagents was first investigated. The addition of 10 μ moles arsenite or iodacetate did not influence the reaction. The combined addition of these substances was also without effect.

A similar addition of the metal inactivating fluoride was also without influence on the reaction rate when this was measured over a period of two minutes. Such an inhibition experiment has been included in the experimental series reported in Figure 6.

The Effect of Nucleotides

The addition of nucleotide coenzymes is not required for the carboxylation of PEP to OAA in *Neisseria meningitidis*.

A more accurate analysis of the effect of such cofactors on the rate of the PEP carboxylase reaction, however, is rendered difficult in the combined spectrophotometric assay by the activity of the nucleotides in other enzyme reactions leading from PEP. Such reactions have previously been demonstrated in the crude extracts under study (Jysum 1960 and Jysum, Borchgrevink & Jysum 1961). Most important in this connection is a coupling of the PEP transphosphorylase with the lactic dehydrogenase. In the presence of ADP meningococcal extracts oxidize DPNH with PEP as the substrate under the formation of lactic acid. This side reaction resulted in a 16 per cent increase in the rate of DPNH oxidation in the combined photometric assay when ADP was added. When ATP was added on the other hand, a small inhibitory effect was noted. Using a similar technique the inosine phosphates had no effect on the reaction rate, a fact which is taken to indicate that these cofactors do not function in any of the enzyme reactions adjacent to the PEP carboxylase.

It is a consequence of these features that the effect of ADP and ATP on the PEP
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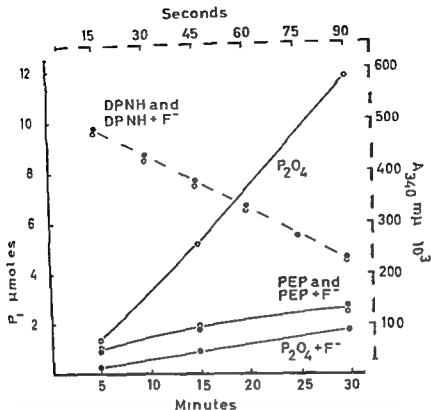


Fig 6

The effect of fluoride on the pyrophosphatase and on the PEP carboxylase in extracts from *N. meningitidis*

In product on from IEP and from pyrophosphate The mixture contained in a volume of 3 ml 75 μ moles tris as buffer pH 7.4 3 μ moles MgCl_2 and 0.5 ml cell free extract dilution. The substrate 10 μ moles IEP or 10 μ moles $\text{Na}_2\text{P}_2\text{O}_7$ was added at zero time. The effect of F was assayed by the addition of 25 μ moles KF.

IEP carboxylase activity The system was the same as the one described in the legend to Fig 2. The effect of F was tested by the addition of 25 μ moles KF.

Open circles: Experiments without F. Filled circles: Experiments with F.

concentrations of inorganic phosphate in their systems accelerated the reaction. The experiments recorded in Table 2 show that no significant increase could be similarly demonstrated in the meningococcal enzyme activity. It is possible, however, that the quantity of inorganic phosphate already present in the crude extracts may be sufficient for an optimal reaction rate. Under the conditions of the experiment this quantity amounted to 0.1 μ mole which apparently is less than that found optimal by the authors mentioned.

It is seen from the data of Table 2 that above a certain concentration in the present experiments approximately 0.0115 M further increase in the orthophosphate concentration brought about a progressive inhibition.

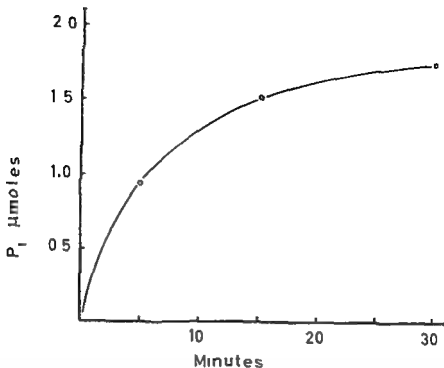


Fig 5

Appearance of inorganic phosphate in the PEP carboxylase reaction. The mixture contained in 3 ml 75 μ moles tris as buffer pH 7.4, 3 μ moles $MgCl_2$, 10 μ moles PEP and 0.5 ml meningococcus cell free extract dilution. After the indicated intervals the reaction was stopped by the addition of 2 ml 10 per cent TCA. The supernatants were analysed for free P_i .

TABLE 2

Effect of Orthophosphate Concentration on the Rate of the PEP Carboxylase Reaction

Orthophosphate added μ moles	0	5	10	30	60	100	150	300
Rate of the Reaction	170	170	175	171	123	109	88	65

The cuvette contained in a volume of 2.6 ml 20 μ moles tris as buffer pH 7.4 (with the exception of the sample with 300 μ moles P_i which contained 13.75 μ moles tris), 3 μ moles KCN, 2.5 μ moles $MgCl_2$, 0.25 μ moles DPNH, 50 μ l malic dehydrogenase (excess), 2 μ moles PEP, 0.5 ml meningococcus cell free extract dilution and P_i as a buffer with pH 7.4 as indicated. The rate of the reaction is given as $\Delta A_{340} 10^3$ in the first 60 seconds of the reaction. DPNH oxidation without PEP is deducted. In the absence of added phosphate the reaction mixture contained 0.1 μ mole free inorganic phosphate. The observed change in the phosphate content due to the reaction was on an average 0.07 μ moles.

The Effect of Inorganic Phosphate

When PEP is converted into OAA in the presence of meningococcus cell free extract, inorganic phosphate appears in the way it may be seen from the data presented in Figure 5. The rate of inorganic phosphate formation is the same whether DPNH and malic dehydrogenase are present or absent.

Tchen & Vennesland (1955) as well as Walker (1957) found that low

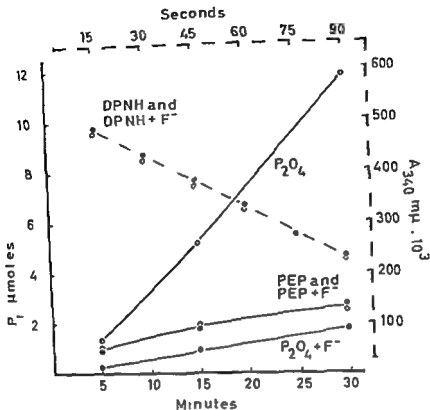


Fig. 6

PEP carboxylase activity. The system was the same as the one described in the legend to Fig. 2. The effect of F^- was tested by the addition of 25 μmoles KF. Open circles: Experiments without F^- . Filled circles: Experiments with F^- .

concentrations of inorganic phosphate in their systems accelerated the reaction. The experiments recorded in Table 2 show that no significant increase could be similarly demonstrated in the meningococcal enzyme activity. It is possible, however, that the quantity of inorganic phosphate already present in the crude extracts may be sufficient for an optimal reaction rate. Under the conditions of the experiment this quantity amounted to 0.1 μmole , which apparently is less than that found optimal by the authors mentioned.

It is seen from the data of Table 2 that above a certain concentration, in the present experiments approximately 0.0115 M, further increase in the orthophosphate concentration brought about a progressive inhibition.

The Rôle of an Inorganic Pyrophosphatase Activity

It may be suggested that the experimental data recorded above result from the coupling of reaction (3) with an inorganic pyrophosphatase activity. Several experiments were accordingly arranged to elucidate this possibility.

It was soon found that a pronounced pyrophosphatase effect was indeed present in meningococcal extracts. This activity has been demonstrated in Figure 6. The pyrophosphatase, however, could be strongly inhibited by the addition of fluoride ions. As seen from the data recorded in the figure a similar addition of fluoride had no significant effect on the PEP carboxylase activity as expressed by the DPNH oxidation. When the production of inorganic phosphate from PEP in the carboxylase reaction was followed such as it has also been illustrated by the curves, no significant effect could be found as the result of an addition of fluoride.

These features point out equation (2) as the one which best explains the experimental data.

Comparison of Adapted and non Adapted Meningococci

Activities of the PEP carboxylase reaction were also compared in cell free extracts from meningococci of the "wild type" and meningococci of the same strain adapted to growth on a minimal medium (Jyssum 1960). In one of these experiments the following activities were found in the spectrophotometric technique

M6 "orig"	(Extr. 2)	63.2	ΔA_{310}	10 ⁴ /min./0.1 mg N
M6 "adapt"	(Extr. 48)	62.9	ΔA_{310}	10 ⁴ /min./0.1 mg N

Thus, no significant change was found in the PEP carboxylase activity after an adaption to growth on a minimal medium with glucose as the only source of carbon and energy.

DISCUSSION

The formation of oxalacetic acid from phosphoenolpyruvic acid and CO₂ apparently is a key reaction in the physiology of *Neisseria meningitidis*. In the absence of a malic dehydrogenase (Jyssum 1960) the reaction becomes a most important link in order to provide the cell with biosynthetic products which draw their carbon skeleton from the tricarboxylic acid cycle intermediates. It also becomes indispensable for an energy yielding oxidation by way of the tricarboxylic and dicarboxylic acids.

When considering a complete, cyclic reaction, however, this becomes highly unlikely because of the unfavourable energy exchange involved in the synthesis of oxalacetic acid from pyruvic acid after the latter has been produced from malic acid by the malic enzyme (Jyssum 1960).

The synthesis of PEP from pyruvic acid by the PFP transphosphorylase (Jyssum Borchgrevink & Jyssum 1961) is far too slow to form PEP at the required rate (Tchen & Vennesland 1966). Furthermore the high energy phosphate bond of PFP is presumably lost when P_i is produced by the PEP carboxylase (Walker 1967).

These theoretical considerations are indeed in agreement with oxidation experiments in Warburg technique (Jyssum Borchgrevink & Jyssum 1961). The "primary oxidative pathway of glucose is rapid and complete while the secondary pathway is incomplete since only approximately half of the acetate which should hypothetically be generated by a primary pathway is completely oxidized to CO_2 and H_2O . That this "secondary route of oxidation involves the intermediates of the tricarboxylic acid cycle has been demonstrated in a tracer technique (Results to be published). It is at present assumed that the tricarboxylic acid cycle reactions cease to function in the oxidation of glucose when no more PFP is synthesized from above. In agreement with this hypothesis is also the finding that lactic and pyruvic acids are not oxidized further down than to the acetate level by suspensions of *Neisseria meningitidis* (Jyssum unpublished results).

In experiments with suspensions of *Neisseria meningitidis* an inhibition by inorganic phosphate was found on the oxidation of glucose and a number of glycolytic intermediates (Jyssum Borchgrevink & Jyssum 1961). This inhibition of the oxidation seems to involve the oxidation of glucose below the acetate level i.e. the secondary oxidative pathway. It may be suggested that this inhibition is a consequence of the phosphate inhibition of the PFP carboxylase described in the present paper.

SUMMARY

The irreversible carboxylation of phosphoenolpyruvic acid to yield oxalacetic acid and P_i has been catalysed by cell free extracts from *Neisseria meningitidis*. The synthesis takes place by way of a PFP carboxylase.

No evidence of other enzymatic formations of OAA from PFP has been found.

The PFP carboxylase is activated by the cations Mn^{++} , Co^{++} and Mg^{++} in the order mentioned. The reaction is inhibited by Cd^{++} , Ca^{++} and Zn^{++} .

The effect of nucleoside coenzymes, P_i and some usual inhibitors on the reaction rate has been investigated.

An inorganic pyrophosphatase which is present in cell free extracts from *Neisseria meningitidis* does not affect the PFP carboxylase reaction described.

The PFP carboxylase has been discussed in relation to the oxidative metabolism of *Neisseria meningitidis*.

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AN IN VITRO GLIOTOXIC EFFECT OF SERUM FROM ANIMALS WITH EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS

By

OLF BERG and BENGT KÄLLEN

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Bornstein & Appel (1961 a) published results demonstrating in serum from rabbits with allergic encephalomyelitis the presence of a factor causing a dissolution of myelin in cultures of neonatal rat cerebellum prepared on reconstituted rat tail collagen. A swelling of the myelin was the first sign of the toxic effect, later resulting in fragmentation of the myelin without damage to the axis cylinders. Cell swelling also affected glia elements, some cells died. This phenomenon was, however, not discussed further. Bornstein & Appel (1961 b) mention that this serum factor is thermostable but needs complement.

Cytotoxic serum factors have been found in patients with some thyroid diseases, noticeably Hashimoto's thyroiditis, capable of killing human thyroid cells in tissue culture (cf. Pulvertaft *et al.* 1959, Irvine 1960, Doniach *et al.* 1961). Also in animals showing homograft reaction, cytotoxic serum factors have been described (cf. O'Gorman 1960, Terasaki *et al.* 1961).

As is well known in tissue culture work, cytotoxicity may be present also in sera from normal individuals. Recently, Bolande (1960) published an account of the different susceptibility of various cell types to cytotoxic factors present in a pool of normal human sera. He was able to show that "normal" cells, *i.e.* cells grown primarily from explants, are relatively resistant to such cytotoxic factors, whereas tumour cells and transformed cell strains show a higher susceptibility. Such toxic factors could be inactivated by heating to 56° for 30 min, and the effect was not reconstituted by normal guinea-pig complement.

In the present paper, sera from animals with experimental allergic encephalomyelitis (EAE) have been tested for possible toxic effect on neonatal glia cells in plasma clot tissue culture.

MATERIAL AND METHODS

Rabbits (weighing about 2.25 kg), guinea-pigs (weighing about 400-800 g) and homozygotic rats (R strain, weighing about 200 g) have been used. IAF has been produced by injection of a mixture of calf spinal cord emulsified in Freund's complete adjuvant (Difco). Rabbits and guinea pigs were injected in the foot-pads usually 3 injections, each of about 0.05-0.1 ml, were given to each animal. In cases of guinea-pigs a good percentage of IAF with distinct clinical signs and typical histological changes were obtained. Rabbits also showed a satisfactory response although the number of sick animals was somewhat lower. In cases of rats injected in the back with 6×0.1 ml of the mixture only few, clear-cut encephalitic changes occurred.

Blood was taken from controls and from experimental animals. Rabbits and guinea-pigs were bled through a carotid artery, rats by cardiac puncture. Usually the animals were bled to death—in only a few cases repeated blood samples were taken. The serum was separated by centrifugation immediately after coagulation of the blood. The serum was stored in deep-freeze until used.

Four guinea pigs were injected with calf spinal cord and incomplete adjuvant. Three guinea-pigs were sensitized with liver and complete adjuvant to serve as further control.

Glia cultures were prepared from neonatal rats. Animals younger than one week were used. Cerebellum and mesencephalon were removed aseptically and placed into pre-warmed Tyrode solution where they were cut into explants measuring about 1 mm square mm. These were placed in Carrel flasks (D 3.5 cm) in a clot consisting of 50 per cent unheparinized adult cockerel plasma, 5 per cent chick embryo extract and 45 per cent Tyrode solution. Next day, a fluid phase was added consisting of 50 per cent adult human serum, 5 per cent chick embryo extract and 45 per cent Tyrode. This fluid was changed once a week but in most cases cultures were used before reaching this age. Good glial growth was often obtained after 4-5 days and the cultures could be used for tests.

A culture with good glia cells was chosen and a still picture was taken using phase contrast microscope. The fluid phase was removed, the culture washed with Tyrode, and the serum to be tested added undiluted. At regular intervals photographs were taken of the same microscopic field. In order to obtain better optical conditions some experiments were made with perfusion chambers.

RESULTS

1. *Gliotoxic Effect in vitro*

In many sera a toxic effect on glia cells was found. In Figures 1 and 2 are shown the typical changes of the cultured cells when such toxic serum had been added. These serial photographs were taken by the aid of a phase-contrast microscope and show glia cells in a perfusion chamber.

The cytotoxic characteristics associated with the gliotoxic effect are swelling of the cytoplasm with retraction of cellular processes, nuclear membrane becomes unusually distinct and the nucleus irregular (Fig. 3), the dying cell is transformed to a globular, strongly light-refracting body. The process of degeneration may stop and a return to normal or nearly normal cellular appearance may take place. Fibroblasts were not affected (Fig. 4).

With some sera a complete, rapid degeneration occurred in nearly all glia cells within 1 hour. This effect was judged as ++. In other cases, with other sera, only few cells degenerated, the degeneration took more than 2 hours, or was reversible. Such sera were judged as +. With yet other sera, finally, no effect on the glia cells were seen. These were judged as 0.

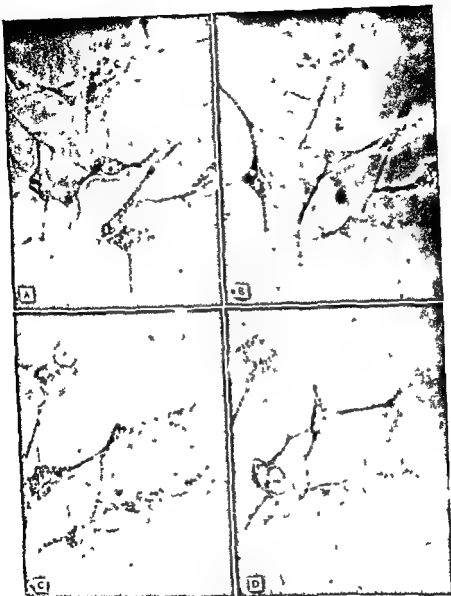


Fig. 1

Fig. 1. A: e-c micrograph taken of a neonatal rat brain culture in a perfusion chamber. B: Effect of addition of FAE guinea pig serum. C: 1 hour after addition of serum from FAE guinea pig No. 16. D: 3 hours after addition of serum. E: 18 hours after addition of serum. In this case a slow degeneration occurred in some of the big ten or glia cells. The effect was judged as +. Magnification 690 \times .



Fig. 2

Phase-contrast microphotogram, taken of neonatal brain culture in a perfusion chamber. A: Before addition of EAF rabbit serum. B: 4 minutes after addition of serum from a rabbit with allergic encephalomyelitis. C: 28 minutes after addition of serum. D: 5 hours after addition of serum--In this case a rapid and nearly complete degeneration of the astrocytes occurred. The effect was judged as ++.

Magnification 730 X



Fig 3

Phase-contrast microphotogram taken of neonatal brain culture in a perfusion chamber Same culture as in Fig 1 Degenerated astrocytes Note irregular nucleus with dense nuclear membrane Magnification 1800 \times



Fig 4

Phase-contrast microphotogram taken of neonatal brain culture in a perfusion chamber Same culture as in Figs 1 and 3 Note degenerated glia cells intermingled with unaffected fibroblasts Magnification 780 \times

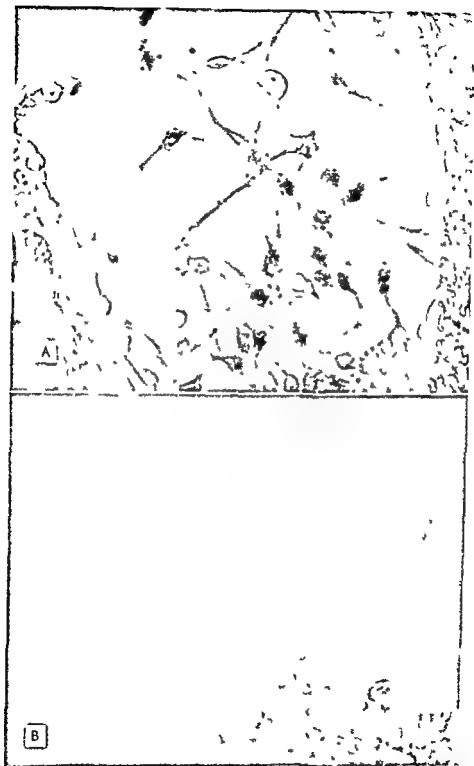


Fig 5

Phase contrast microphotogram taken of neonatal brain culture in a barrel flask
 A Before addition of control rabbit serum B 50 minutes after addition of serum
 Note complete dissolution of the clot with detachment of all cells due to fibrinolytic
 activity of the serum Magnification 380 X

The present authors do not wish to try and grade the gliotoxic effect more closely. Slight variations in the response of various cultures to the same serum were sometimes seen. The above mentioned classification is, however, found justifiable.

2 Unspecific Degeneration Phenomena in the Cultures

Using some sera—both normal and EAE—an extremely rapid complete dissolution of the clot with cells was seen. An example is shown in Fig. 5. In these cases an obvious fibrinolysis occurred. All cells were detached and died. This effect, which may be associated with an abnormal plasmin activity of the sera tested, could be distinguished very easily from the specific gliotoxic effect as described above. It was seen only in some of the rabbit sera, never in the guinea pig or rat sera. The effect could be partially arrested by the addition of lysin ethyl ester—a plasmin inhibitor.

In all of the cultures some degenerating glia cells could always be found—healthy cultures with a minimum of such cells were in all cases chosen for the tests.

3 Gliotoxic Activity in Various Types of Sera

Guinea pig. Sera from 8 control animals were tested. In one of these a weak reversible effect was seen, judged as +. The other 7 cases were judged as 0. Sera from 3 guinea pigs injected with liver and complete adjuvant were negative.

TABLE 1
Results from Tests of Gliotoxic Activity in Sera from Guinea Pigs with EAE

Animal number	Days after injection	Clinical signs	In vitro effect	Note
H	17	0	0	
B	19	+	(+)	
	35	0	++	
13	23	0	0	Positive histology
15	24	0	0	
	45	0	0	Positive histology
14	24	0	0	
	50	+	++	
N	14	+	+	
O	15	+	++	
I	12	++	++	
11	13	++	++	
10	11	++	++	
F	16	++	++	
V	16	++	++	
L	11	++	++	
M	11	++	+	
P	19	++	++	
12	18	+	0	
	23	++	0	
16	14	++	+	

Sera from 17 guinea-pigs injected with spinal cord and complete adjuvant were tested. The results are shown in Table 1, where also some data on the animal material are given. Sera from 4 animals injected with spinal cord and incomplete adjuvant were all negative.

Rabbit As mentioned above, many rabbit sera—both control and EAE—showed the unspecific fibrinolytic effect and no gliotoxicity could be judged. In some cases, however, no fibrinolysis occurred, a gliotoxic effect in EAE sera here being observable (cf. Fig. 2).

Rat Sera from 4 rats with EAE were tested; 3 were negative, in the fourth a faint, reversible effect was seen. No gliotoxic activity was seen in 4 normal rat sera.

4 Heat Inactivation

Heating the EAE serum to 56° C for half an hour eliminated the toxic effect of the serum. Adding unheated normal guinea-pig serum did not restore the effect. This condition agrees well with the description of Bolande (1960) and Pulvertaft *et al.* (1959), but is opposed to that of Irvine (1960) and Bornstein & Appel (1961 b).

DISCUSSION

A gliotoxic effect has been demonstrated in sera from animals with experimental allergic encephalomyelitis, noxious to neonatal glia cells in tissue culture. It resembles the cytotoxic phenomenon observed in sera from patients with Hashimoto's disease, and it may be closely related to the myelinolytic factor described by Bornstein & Appel in sera from rabbits with EAE.

The gliotoxic factor can be demonstrated in sera from guinea-pigs and rabbits with EAE, but not with certainty in our small series of rats with EAE, possibly because in the latter case homologous sera were used. It may also be the result of differences in the immunologic response of the various animal species.

In our series of guinea-pigs no absolute correspondence was found between the cytotoxic serum activity *in vitro* and the clinical status of the donor animal. At least in one case (No. 12) a clinically marked encephalomyelitis was present without any cytotoxic activity *in vitro*. In one case (No. B at 35 days) a marked cytotoxicity was present, but the clinical signs of the disease had regressed. In one normal animal a faint cytotoxicity *in vitro* was found. Waksman (1959) and others have shown that the level of complement-fixing circulating antibodies against brain is not correlated to the encephalomyelitic process. On the other hand, in Hashimoto's disease the level of circulating complement fixing antibodies and the cytotoxic activity are not completely parallelled.

The significance of the cytotoxic factors *in vivo* is uncertain. So far efforts to transfer experimental allergic encephalomyelitis with serum

have failed. A review is given by Chase (1959). The *in vitro* gliotoxic factor is effective and constitutes a parallel to similar phenomena in other immunization processes. It may, however, be only part of the complex antibody spectrum developed in experimental allergic encephalomyelitis and without a pathogenetic significance.

SUMMARY

In sera from guinea pigs and rabbits with experimental allergic encephalomyelitis a factor is found, selectively toxic for glia cells cultured *in vitro* from neonatal rat brain substance. The presence of the gliotoxic factor is not directly correlated with the clinical status of the animals. It has not been demonstrated with certainty in sera from rats with experimental allergic encephalomyelitis.

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The significance of the cytotoxic factors *in vivo* is uncertain. So far efforts to transfer experimental allergic encephalomyelitis with serum

— — — — — modification of Ouchter
 1 mm thick (Rheinagar
 slide Holes about 15 mm
 gel The holes were filled

with the antigen preparation (brain or spinal cord extract) or with solutions to be examined for antibodies against the antigen extract The plates were placed in a moist chamber for 2 days during which time antigen antibody reactions may develop Excess protein was then eluted and the slide was dried The preparations were stained with amido black 10 B (for proteins) and Sudan black (for lipoproteins)

Immuno electrophoresis was performed at pH 8.9 and ionic strength of 0.05 by Heremans modification (1960) of Scheidegger's micromethod (1955) which is based on Grabar & Williams' original method (1953) The technique staining procedure etc has been described in a previous paper (Dencker & Swahn 1961)

In the experiments using precipitation in agar gel as well as in those with immu-

nology solution was applied

RESULTS

Immune precipitation in agar gel (modified Ouchterlony technique)

Figure 1 shows a slide in which the central hole was filled with the antigen prepared from calf spinal cord and the other holes with FAE serum and with sera from two patients with multiple sclerosis Four precipitation lines are seen in the area between the central hole and the hole containing FAE serum In an attempt to ascertain whether any of these lines were due to a protein specific for nervous tissue a second experiment was performed in which the serum was absorbed with normal calf plasma Antibodies against non specific antigens are then removed Figure 2 illustrates the result Only one of the four precipitation lines is seen This precipitate does not contain sudanophilic material A diffusion test with calf spinal cord against calf plasma gave no precipitate

When one of the holes was filled with FAF serum and the other with human brain antigen no precipitation lines were obtained

Sera from control guinea pigs and rabbits from guinea pigs sensitized with calf spinal cord and incomplete adjuvant and from a rabbit not developing encephalomyelitis after sensitization with calf spinal cord and complete adjuvant gave no precipitation with calf spinal cord antigen or with human brain antigen

Sera from four patients with multiple sclerosis at varying stages were studied with the precipitation in agar gel method against human brain extract as antigen No precipitate could be demonstrated

Micro Immuno Electrophoresis

The proteins in a calf spinal cord extract were separated by electrophoresis after which the middle groove was filled with FAE serum The electrophoretically separated proteins i.e. the antigens were thus allo-

STUDIES ON SERA OF ANIMALS WITH EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS AND PATIENTS WITH MULTIPLE SCLEROSIS USING IMMUNE PRECIPITATION IN AGAR GEL AND IMMUNO-ELECTROPHORESIS

By

OLL BERG and SVEN J. DENCKER

Received 17 VIII 61

Experimental "allergic" encephalomyelitis (EAE) can be produced in animals by intracutaneous injections of brain substance emulsified in Freund's complete adjuvant. Symptoms appear 2-4 weeks after the injection and then the complement fixation reaction is positive (Hill 1949, Lumsden 1950). Sera from these animals contain a toxic factor, noxious to glia cells in tissue culture (Bornstein & Appel 1961, Berg & Kallen 1961a). A toxic effect on glia cells *in vitro* has also been found in sera from patients with multiple sclerosis (Berg & Kallen 1961b). These reactions have been tentatively ascribed to an assumed formation of immune bodies against nervous tissue. This paper is concerned with an attempt to demonstrate the supposed antibodies by precipitation in agar gel and by immuno-electrophoresis. Organ-specific antibodies against nervous tissue have been demonstrated by Ross (1960) with precipitation in agar gel in rabbits, immunized against rat brain.

MATERIAL AND METHODS

EAE was induced in the usual way in rabbits by intracutaneous injection of a mixture of homogenized calf spinal cord and Freund's complete adjuvant (Difco). Three injections of each 0.05 ml was given to each animal on one occasion. The sera from such animals are referred to as EAE sera.

In the immune precipitation and immuno-electrophoresis experiments calf spinal cord and brain substance taken at autopsy from a patient who had no neurologic disease have been used as antigen. These preparations were homogenized and carefully mixed with the buffer used at immuno-electrophoresis. In such a suspension the electrophoretic mobility of the proteins and their diffusion in agar gel are satisfactory. The sera from multiple sclerosis patients were fresh and used within one hour after collection. In the absorption tests normal calf plasma was used.

Sera from guinea pigs sensitized by injection of a mixture of homogenized spinal cord and Freund's incomplete adjuvant were also studied. These animals did not develop encephalomyelitis. One rabbit in which encephalomyelitis did not appear despite sensitization with spinal cord plus Freund's complete adjuvant was also examined. All sera not used in the fresh state were kept deep frozen (-20°C).

for the precipitation in agar gel, only one of these fractions appeared, namely the one in the β_1 -area (Fig. 4)

COMMENTS

In sera from animals with EAE, sensitized with calf spinal cord, our investigations have demonstrated immune bodies directed against calf spinal cord antigens. When the calf spinal cord antigen is replaced by human brain antigen, no precipitates are formed. The immune bodies demonstrated are therefore probably species-specific.

Ross (1960) showed with an agar gel precipitation technique that two antigens exist, precipitated by EAE serum: an organ-specific one of a lipid nature and a species-specific one of a non lipid nature. The lipid antigen can diffuse only after trypsinization of the brain or spinal cord extract. The species-specific immune bodies, found in the present investigation, may therefore be directed towards brain protein antigens. Other investigators (Hill 1949, Lumsden 1950) have demonstrated complement fixing antibodies in EAE sera. These are organ specific but not species specific, i.e. directed towards nervous tissue irrespective of species. They further showed that the antibody titer was not correlated to the severity of the encephalomyelitis. Sensitized animals without clinical signs of the disease may show a high titer. This fact may indicate that the antibodies in question are not pathogenic. It is therefore important to investigate the possible pathogenic significance of the species specific antibodies.

The negative results of examinations of the four multiple sclerosis sera agree with the absence of complement fixing reactions in such sera (Stauffer & Waksman 1954). Definite conclusions cannot, however, be drawn until a larger material has been studied and methodological variations have been tried.

SUMMARY

Antibodies against central nervous system tissue in sera from animals with experimental allergic encephalomyelitis have been demonstrated with a precipitation in agar gel method according to Ouchterlony. The antibodies are apparently species-specific. Immuno-electrophoretically the nervous tissue antigen migrates like a β_1 -protein.

No immune bodies to human brain tissue have been demonstrated in sera from four patients with multiple sclerosis.

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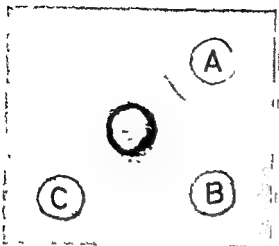


Fig 1

Fig 1 The central hole contains calf spinal cord extract, the other holes contain serum from a rabbit with IAF (sclerosis (B and C) Antibodies

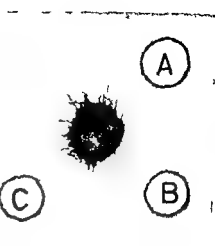


Fig 2

Fig 2 Same experimental conditions organ-specific antibodies with calf plasma Only one precipitation line is seen

Fig 3



Fig 3

Fig 3 Immuno electrophoresis of calf spinal cord (in hole) against IAE rabbit serum (in middle groove) Three fractions appeared one at the site of albumin the other in the $\alpha_2 \beta_1$ field

Fig 4 Same experimental conditions as in Fig 3 but after absorption with calf plasma Only the precipitate in the β_1 area is now seen

wed to diffuse towards the antibodies, possibly present in the EAE serum Figure 3 shows such a preparation where three protein fractions can be seen One of the proteins was demonstrated at the site of albumin, the other two were unknown fractions in the $\alpha - \beta_1$ -field When the EAE rabbit serum was absorbed with calf plasma in the way described

PREPARATION OF COMPLEMENT REAGENTS BY MEANS OF GEL FILTRATION

138

K.-E. FJELLSTROM

Received 5 ix 61

The four known complement factors in serum are usually determined by titration with reagents, called R1, R2, R3 and R4, which lack the factors C'1, C'2, C'3 and C'4 respectively.

In this communication a new method for the preparation of R1 and R2 is described. Serum is passed through a column consisting of granules of cross-linked dextran gel (Sephadex® G 25). In this way fractions well suited as R1 and R2 reagents are obtained. The method is quick and gives more reliable results than other procedures used hitherto (1, 2).

MATERIAL AND METHODS

See

The reagents were prepared from freshly drawn human serum pooled from 10–15 donors.

Demonstration of Complement Activity

Washing and sensitization of sheep blood cells were carried out as described by Kabat & Murrer (3). Haemolysis was estimated after 30 minutes at 37° C in a total volume of 1.25 ml. containing 1 ml. of

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Implement Reagents

113 and 114 were prepared by treating serum with zymosan and hydrazine reagents respectively (3, 6, 7). All the reagents, namely, that they were not anticom-

This investigation has been supported by grants from the Medical Faculty of the University of Uppsala and the Regnell Fund. Material was kindly supplied by AB Pharmacia and the Department of Bacteriology.

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natants with low pH and low ionic strength were adjusted to pH 6.75-7.0 with 0.1 M NaOH and to blood isotonicity with 1.5 M saline.

The precipitate was washed twice with the acetate buffer, and was then dissolved in veronal buffer. For washing it is important that the particles of the precipitate should be as fine as possible, this is most easily attained if they are suspended in the smallest convenient volume of washing buffer and are broken up with a glass rod or wooden stick before the final buffer volume is added (cf. *Ecker et al.* (7)).

Preparation of R1 Reagent

Table 1 summarizes an experiment in which a gel volume of 88 ml was used, to which serum volumes, varying from 4 ml to 12 ml, were applied. In this case both the fractions without precipitates and the supernatant fluids from fractions in which the euglobulins had been precipitated were collected. It was found that if the serum volume was small, the pH of all the fractions remained at about 5.4-5.7. When the quantity of serum was increased, a large part of the fractions eluted later had a higher pH (between about 7 and 8).

TABLE 1
The Properties of the Supernatants from Different Serum Volumes
Filtered through Sephadex G 25

Experiment no.	Serum volume ml	Fraction volume ml	Fraction no.	pH of the supernatants	Content of C1	Accepted as R1
1	4	2	1	5.5	0	yes
			2	5.5	0	
			3	5.5	0	
			4	5.7	0	
2	6	3	1	5.4	0	yes
			2	5.8	0	-
			3	6.9	tr	
			4	7.1	0	
3	8	4	1	5.4	0	yes
			2	6.1	0	yes
			3	7.2	+	
			4	7.5	+	
4	10	5	1	5.5	0	yes
			2	7.3	+	-
			3	7.8	+	
			4	7.7	0	
5	12	6	1	5.8	0	yes
			2	8.0	+	
			3	8.2	0	
			4	8.4	0	

Column ID = 20 mm Height 28 cm Gel volume 88 ml
Equilibration and elution with acetate buffer of pH 5.4 μ 0.02

in a sublytic dose of total complement by at least 200 per cent (8) All glassware used had been cleaned in dichromate sulphuric acid

Quantitative protein determinations were carried out according to a modified Folin method (9)

Gel Filtration

Principle

Sephadex ®1 consists of cross-linked dextran molecules The powder, while insoluble in water, is hydrophilic, and swells to a gel with a considerable increase in volume It is furthermore non ionic and its polar properties depend on its abundant content of hydroxide groups (10) According to Porath & Flodin (10-11) the gel acts predominantly as a molecular sieve, the separation of components being determined by their molecular size so that smaller molecules are retarded more than larger ones when passing through a gel column Large molecules such as those of proteins are not retarded at all because they are unable to penetrate into the gel particles Small molecules such as electrolytes which can diffuse into the gel particles thus elute more slowly When a serum is filtered through a gel which is equilibrated with a buffer of lower ionic strength than that of the serum the serum electrolytes are retarded and the serum proteins gradually pass into a medium of a lower and constant ionic strength

If, however, the ionic strength of the equilibration and elution buffer (in the following called acetate buffer) is sufficiently low the less soluble protein of the serum (the euglobulin) precipitates in the column This offers the possibility of a rapid separation of euglobulins from the more easily soluble pseudo globulins and albumin The quantity of precipitate formed is dependent on the relation between the volume of the serum sample and that of the column The pH of the acetate buffer is also of great importance for both the quantity of the precipitate formed and for its properties

Equilibration and Packing of the Column

For a gel volume of 100 ml approximately 35 gram of dry substance Sephadex G 25 is required This is stirred in a 400 ml beaker containing acetate buffer ionic strength 0.02 This buffer was chosen because of the experiments reported by Ecker *et al* (7) on the preparation of R1 and R2 by dialysis of serum When most of the gel has sedimented the smallest particles are still freely suspended and can be removed Packing of the column was done as described by Flodin (12) and Porath (13)

EXPERIMENTS

General Remarks

The investigations were planned with a view, *inter alia*, to elucidating the experimental conditions most advantageous with respect to the preparation of R1 and R2

Gel filtration was carried out at room temperature and collection of fractions was started when protein first appeared in the eluate which can be detected for example by the Tyndall phenomenon in the trans-illuminated eluate At first only dissolved protein appears At different times thereafter depending on several factors not treated here, precipitated protein appears together with dissolved protein

In those fractions which contained both easily soluble and poorly soluble protein, the precipitate was separated from the soluble protein by centrifugation at 3000 rpm for 5-10 min The pH and the conductivity of the supernatant fluids so obtained were measured Super

1 From Pharmacia Ltd Uppsala Sweden

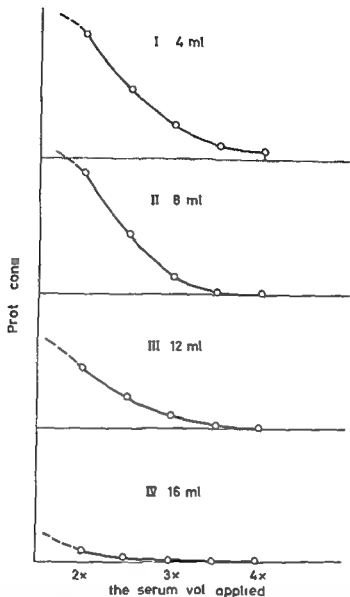


Fig 1

Gel filtration Elution of serum proteins in relation to applied serum volume. Filtration on Sephadex G 25. Gel volume 88 ml. Equilibration and elution with acetate buffer of pH 5.4 μ 0.02. Abscissa: Eluate volume = times volume of applied serum. Ordinate: Relative protein concentration (Folin optical density at 500 m μ , Beckman B). In four experiments 4, 8, 12 and 16 ml serum was applied to the column and eluted. The last part of the protein curve is illustrated. The optical density is measured in fractions with volumes equal to half the volume of serum. The curves show that relative to the serum volume applied larger volumes are eluted proportionally more rapidly than smaller volumes.

Only with the smallest serum volume of 4 ml were all fractions completely liberated from C'1, but only fraction 2 in this experiment fulfilled the R1 criteria, because the protein was too dilute in the other fractions. If the serum quantity was increased, the gel was unable to precipitate all the C'1 out of the serum. It is true that in experi-

Because of this marked pH dependence of the fourth component the influence of the pH of the acetate buffer was investigated (Table 3). It was found that C'3 and to some degree C'4 increases in the supernatants parallel to increasing pH of the buffer, while in the precipitates the reverse effect is observed. Furthermore, there is a drop of C'4 to zero when a pH of 5.75 is used.

The influence of the temperature has not been studied. Since the solubility of the euglobulins varies directly with the temperature, it should be an advantage to carry out the gel filtration in the cold, but from the complement activity aspect the yield was fully satisfactory even at room temperature.

Fractions with low protein concentrations, which are eluted towards the end of the process, can exhibit anticomplementary effects.

Columns sometimes pack together and become more compact, so that the flow rate decreases considerably and the filtration time becomes unnecessarily long. If this occurs the column can be regenerated first with 0.1-0.3 N NaOH and then with 0.5 M NaCl, after which the gel regains its original filtration properties. The effluent obtained during such a regeneration was tested for the presence of protein by Folin's method; this showed that the adsorption due to protein was less than 0.5 per cent of a filtered serum quantity of 80 ml. If bacterial growth occurs in the column, it should be repacked with new substance.

Proposed Methods for Preparation of Reagents R1 and R2

The recommendations apply under the assumption that Sephadex G 25 and columns with ordinary relationships between inner diameter and height are used. Another condition is that fresh serum with a pH of about 8.0 be used. Serum which has been frozen by solid carbon dioxide can decrease in pH considerably below 7.0, and this must then be adjusted before the filtration.

Preparation of R1

Equilibration and elution with acetate buffer pH 5.4-5.6, μ 0.02.

The ratio between serum volume and gel volume should not exceed 0.13. If, for example, the gel volume is 80 ml, the quantity of serum applied should not exceed 10 ml, and the best result is obtained if the actual volume loaded is only 60-70 per cent of this upper limit. When protein begins to appear in the eluate, 3-4 fractions are collected, the volume of each is equal to about 0.6-0.7 times the serum volume applied. After precipitate, the pH is adjusted to 15, after which each fraction is tested. R1 and R4. As a rule adequate R1 reagents are obtained in fraction 1 or 2 or these two fractions combined.

1.6–1.8 corresponding to an ionic strength of 0.02. The ratio serum volume/gel volume in this case is approximately 0.13. With serum volumes below 12 ml, satisfactory R2 was obtained from 10 ml serum, while R1 had an insufficient titre for the three requisite components C'2, C'3 and C'4. With serum quantities over 12 ml the supernatant was not completely free from C'1, and the precipitate contained too little of the necessary components for R2. This was due to an increase of pH and ionic strength in the effluent and a quantitative decrease of the amount of the precipitate. With the help of the above mentioned ratio the optimal quantity of serum for another column with the gel volume 141 ml was calculated to be 20 ml. Experiment 7, Table 2 confirmed the results with the 88 ml gel volume and shows that here also the total protein eluate had a pH of approximately 6.2 and that satisfactory R1, R2 reagents were prepared.

It may thus be seen in Table 2 that under certain conditions R1 and R2 can be simultaneously obtained from a single filtration procedure.

The flow rate does not appear to influence the results within fairly wide limits (experiment 3–4, Table 2). It is apparently immaterial whether the precipitate is centrifuged down immediately after the gel filtration or allowed to remain in the eluate for a few hours at +4° C before centrifugation and washing (experiment 4, Table 2).

On the other hand experiment 8, Table 2, shows that the pH of the buffer in which the precipitate is washed is of importance. In this case the precipitate had been divided into two equal portions, one of which was washed with acetate buffer of pH 5.4 and the other with buffer of pH 6.2. On washing at pH 5.4 a good R2 was obtained, but at pH 6.2 a large part of C'4 disappears and this R2 cannot substitute R4. In another experiment the precipitate was washed with buffer of 6.7. In this case all C'4 activity disappeared. C'4 was found in the washing buffer in a high titre, together with some C'3, but the precipitate, however, was able to substitute R3.

TABLE 3

The Effect of Different Buffer pH on the Yield of Complement Components

pH in the equilibration and elution buffer		0%, hemolysis units				pH in supernatants
		C'1	C'2	C'3	C'4	
5.1	supernatant	0	200	24	50	5.2
	precipitate	115	0	17	43	
5.4	supernatant	0	240	12	52	5.7
	precipitate	125	0	18	32	
5.75	supernatant	0	162	59	13	6.6
	precipitate	125	0	31	0	

Gel Filtration Sephadex G 20; gel volume 84 ml. Equilibration and elution of the gel with acetate buffer μ 0.02, pH 5.1, 5.4 and 5.75 respectively. 10 ml serum applied. 20 ml effluent was sampled in each experiment.

theless it cannot be excluded that the precipitate is entrapped within the column. These observations are to be published in detail elsewhere. According to present experience the protein retained in the column does not exceed 0.5 per cent of the total protein applied to the column. Even if this is a relatively small amount it cannot be excluded that it has some significance in connection with the preparation of such minor and specific serum protein components as those related to the complement system.

As the experiments have shown this procedure is well suited for preparation of R1 and R2 in so far that C1 can be quantitatively precipitated with the euglobulin fraction while C2 is retained in the supernatant. Under the conditions chosen C3 and C4 are partly precipitated and partly retained in solution. The method of collecting the protein eluate fractionally almost always gave an insufficient yield of C4 in the precipitate. This difficulty in preparing R2 can most easily be overcome by collecting the eluate in one single fraction which gives a higher yield of C4. The explanation for this evidently is that when the eluate is collected fractionally only the first fraction will have a pH sufficiently low (6.2 or less) to give a precipitate rich enough of C4 (see Table 1 exp. 4 and 5).

Seifler *et al.* (14) found that C4 was the least stable complement component in the pH = 5.4— μ 0.02 precipitate. The experiences from washings of the precipitate with buffers of different pH also support the view that C4 is the complement factor in the precipitate which is the most sensitive to even small physico-chemical changes.

The essential advantage of gel filtration lies in the fact that this can be carried out in a very short period of time, the risk for changes such as denaturation of the proteins thus being reduced to a minimum. The previous method of production of R1 and R2 by dialysis of serum in the cold against an acid buffer with low ionic strength for 24–48 hours gives good results only rather randomly, while the method described here yields good reagents almost without exception.

SUMMARY

A new method for the preparation of reagents R1 and R2 for complement titration has been described. Gel filtrations by means of Sephadex G20 and an elution buffer of low ionic strength and pH has been used in order to precipitate C1 in the euglobulin fraction leaving C2 in the supernatant pseudoglobulin fraction. In this way the time necessary for preparation can be brought down to one hour. The reagents obtained regularly fulfil the criteria for adequate R1 and R2.

Preparation of R2

Equilibration and elution with acetate buffer pH 5.1-5.4, μ 0.02

As in the case of the R1-preparation the serum volume is chosen from the ratio serum volume/gel bed volume, which for the R2-preparation should be about 0.13. The volume of the collected eluate should be 2-3 times the serum volume, and is collected in one sample, mixed by shaking, and centrifuged for 10 min. at 3000 r.p.m. The supernatant is sucked off as completely as possible. The precipitate is washed twice with the acetate buffer. In the final washing the suspended precipitate is divided into two equal portions. The first portion of the precipitate is dissolved in a relatively small volume of veronal buffer, e.g. 1/7-1/5 of the applied serum volume and is tested for the occurrence of C'2 and for the ability to substitute R1, R3 and R4. Subsequently the remaining precipitate portion is dissolved in a suitable volume of buffer depending on the result of the first test. All the procedures for the preparation of R1 and R2 take about one hour.

DISCUSSION

The passage of water, electrolytes and proteins through the Sephadex gel column has been described earlier (10, 13, 15) and treated theoretically as well, but up to now nothing has been known about how the protein precipitated behaves in this connection. It is quite clear that this new method of separating pseudo- and euglobulins is to be studied from physico-chemical and protein-chemical aspects. It is also possible that the results achieved in this investigation have to be modified in some details when such investigations have been performed. However the present author has found his results from preparation of complement reagents of such general interest that they should be presented even if the theoretical background of the method is not yet thoroughly investigated.

As to the precipitation of euglobulin within the column the following is evident from the investigation hitherto performed: Precipitation occurs gradually as serum protein enters a medium with reduced ionic strength and pH and the precipitate remains in the space outside the gel granula and during elution it moves there together with dissolved protein. In the beginning the granulation of the precipitate is fine and increases gradually. Because of the short time for elution the particle size of the precipitated protein will not increase very much. The main effect to be expected from such an increase should be an impeded passage of the precipitate within the column. For example if the column is packed with small gel granula the elution time will be long and result in a delayed appearance of the precipitate in the eluate. The same effect is observed if the elution is interrupted for some time in a column packed with larger gel granula. As evidence serves that under special conditions some adsorption of protein to the gel occurs (cf. 16) Never

THE AETIOLOGY OF RESPIRATORY DISEASE IN MILITARY RECRUITS

3 *Correlation between Clinical Findings and Association of Virus and Bacteria in Infection*

By

Z DINTER, H EKELLUND, G LAURELL, G LINDBOM,
G LÖFSTRÖM, L PHILIPSON and T WESSLÉN

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The first two articles in this series (1 and 2) were concerned with the virological and bacteriological findings in three periods of investigation during the years 1959–1960 in cases of acute respiratory infection at a military camp in Uppsala

The object of the present article is to analyse these cases from a clinical standpoint based on findings of pathogenic micro organisms

The term acute respiratory infection covers a variety of clinical symptoms and signs of diverse aetiology. The clinical picture of the aetiotologically distinct conditions is rarely only characteristic enough as to allow for an identification of the causative organism. Certain infective agents however, may produce a fairly specific set of symptoms, such as seen in influenza in some adenovirus infections, and in streptococcal tonsillitis. During recent years a number of new species of virus have been identified, and difficulties involved in the establishment of diagnoses on the basis of the clinical findings have become more and more accentuated. This is especially true of cases occurring outside isolated communities. Investigations carried out within institutions such as day nurseries and military camps usually give better results, sometimes the aetiology of up to 90 per cent of the clinical cases may be established.

An analysis follows of the material accumulated in the autumn of 1959, the autumn of 1960 and the winter of 1960. The results obtained from isolation of infecting organisms and from serological tests (1, 2) are correlated to the clinical findings. A certain clinical classification

is known as upper respiratory infection

1) *Exudative pharyngitis*. The main criteria are inflammation, lymphoid hypertrophy, and pharyngeal exudate. In addition pyrexia and cervical lymphadenopathy may occur.

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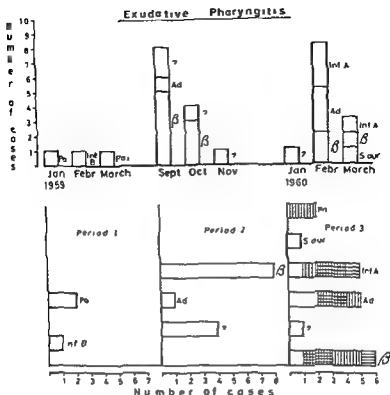


Fig 1

Distribution of cases on the different months of the year correlated to aetiological confirmed infections

- ☐ Pure virus or bacterial infections
- ☒ Bacterial complication of primary virus or other bacterial infection
- ☒ Virus complication of primary bacterial or other virus infection
- ☒ Virus or bacterial infection complicated by more than one other infective agent

Inf A — Influenza A
 Inf B — Influenza B
 Inf C — Influenza C
 Ad — Adenovirus
 Pa — Para influenza virus
 Orn — Ornithosis
 Mon — Mononucleosis
 Hinf — Haemophilus influenzae
 — Beta haemolytic streptococci
 Pn — Pneumococci
 Saur — Staph aureus
 ? — Aetiology unknown

2) *Pharyngo-conjunctivitis* The main criteria are conjunctivitis with follicular hyperplasia (unilateral or bilateral) and pharyngitis (exudative or nonexudative) Pyrexia is not a condition Coryza and constitutional symptoms may be present

3) *Acute respiratory disease (ARD)* The term embraces the syndrome of Dowling *et al* (3) and also the influenza syndrome described by these authors Since we find it impossible on the basis of the history and clinical findings to distinguish between these two syndromes, they have been combined under a single head *Main criteria* Temperature over 38° C, often higher, onset sudden or slow Pharyngitis, with injection, dysphagia and lymphoid hypertrophy of the tonsils, but no exudate, vesicles, or ulcers Constitutional symptoms such as malaise, headache, and muscular pains

Coryza and enlargement of the cervical glands may also be present

4) *Minor respiratory illness (MRI)*, comprising undifferentiated upper respiratory infection (URI) and afebrile acute coryza (common cold) *Main criteria* Temperature below 38° C, pharyngitis with slight redness but no exudate, vesicles, or ulcers, laryngitis or bronchitis with hoarseness and cough, constitutional symptoms absent or mild

RESULTS

The results of the bacteriological and virological investigations are arranged according to the clinical groups The condition required to make an aetiological relationship acceptable was a significant increase in the antibody titres during the course of the illness (cf. earlier papers in the series) On the other hand, the simultaneous isolation of micro-organisms responding to the various serological systems was not considered essential

Exudative Pharyngitis

The findings in this group are collected in Fig 1 Here as in other figures the cases are recorded under two heads, 1) the infective agent considered the primary cause of the illness, and 2) agents present at the same time or complicating the primary infection Double infection and secondary complications are indicated in the figures It follows that the number of infective agents noted will exceed the number of cases

During the *first period* 3 cases occurred, para influenza was considered associated with the disease in January and March, and influenza B in February Influenza B, which predominated during this period, thus produced this clinical picture only in one case

During the *second period*, autumn 1959, the beta-haemolytic streptococci predominated as causative agent in exudative pharyngitis, being responsible for 8 cases, viz 5 in September and 3 in October A further 5 cases were noted, one of these being ascribable to adenovirus types 7 and 4 was of unknown origin

Pharyngo conjunctivitis

This group is illustrated in Fig 2, and comprises 28 cases. The aetiology was confirmed in 19 cases.

During the first period 13 cases occurred. Influenza B predominated (8 cases, 7 of which were uncomplicated and one showed simultaneous infection with pneumococci). One case of para-influenza and one beta haemolytic streptococcus infection occurred, and in three cases the aetiology remained obscure.

During the autumn of 1959 four cases were seen, one of beta haemolytic streptococcal infection, three remained undiagnosed.

During the winter of 1960 7 cases of influenza A occurred. Three were uncomplicated, three had concomitant adenovirus infection, one of these being infected also by pneumococci, the seventh case was complicated by beta haemolytic-streptococcal infection. One case of pharyngo conjunctivitis was caused by adenovirus type 7, and in three cases the aetiology remained unconfirmed.

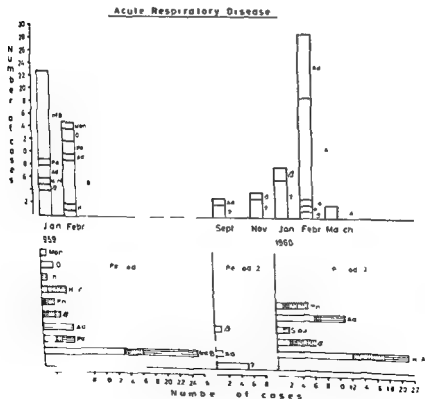


Fig 3

Distribution of cases in the different months of the year correlated to aetiological confirmed infections. For abbreviations see Fig 1.

During the *third period* 12 cases were seen. Of these, 5 were uncomplicated, one case of influenza A, one due to *Staph aureus*, one caused by adenovirus type 7, one of adenovirus infection confirmed only by serological tests, and one due to beta-haemolytic streptococcus infection.

4 cases were seen of complicated influenza type A. In two of these an untyped adenovirus and beta-haemolytic streptococci were seen in addition to this virus. In one case influenza-A virus and beta-haemolytic streptococci were involved. In the fourth case beta-haemolytic streptococci were isolated from both throat swabs, and the antistreptolysin titre was raised in the second blood sample, influenza-A antibodies did not appear until blood was sampled for the 3rd time, however, after the recruit had been in the military sick bay for 16 days, beta-haemolytic streptococci seem to have been the primary cause of illness, the influenza infection possibly being nosocomial.

An infection due to adenovirus type 7 developed parallel with rise in antipneumolysin titre. In another case a beta-haemolytic streptococcal infection was observed, but at the same time a rise in antipneumolysin titre occurred without isolation of the pneumococci. In one case the etiology remained unexplained.

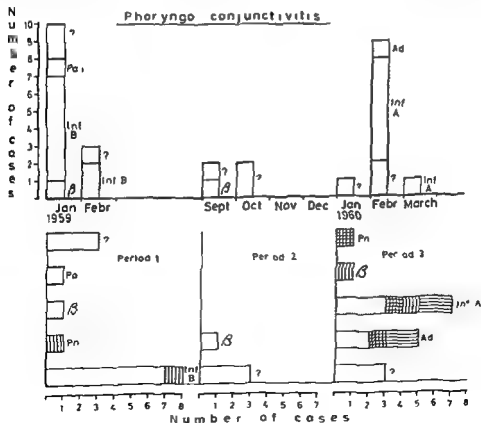


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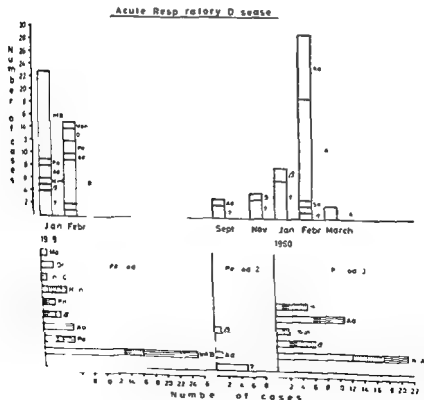


Fig 3

Distribution of cases in the different months of the year correlated to aetiological confirmed infections. For abbreviations see Fig 1.

Acute Respiratory Disease

This was the largest group, including a total of 84 cases. The aetiology was confirmed in 81 %, as can be seen from Fig. 3.

38 cases occurred during the winter of 1959. A significant rise in influenza-B-antibody titre was noted in 25 cases, 13 of which were uncomplicated virus infections. 12 of the cases presented influenza-B-virus infections in various combinations with bacterial or other virus infections.

Adenovirus occurred in combination with influenza-B virus in four cases. Adenovirus type 3 was demonstrated in three of these, a serological diagnosis was obtained only in the fourth. Rise in influenza-B-virus and adenovirus antibody titre occurred simultaneously in two of these cases, where a double infection presumably was present.

In the other two cases the rise in antibody titre to adenovirus preceded that to influenza-B virus. One of these started with general malaise, headache, joint and muscle pains, coryza, hoarseness, sore throat, and a temperature of 38.4° C. Adenovirus type 3 was isolated at the onset of the illness and again 5 days later. The patient was discharged from the sick bay after 10 days of illness, at which time he presented raised adenovirus-antibody titre. He still had coryza and hoarseness, however. After a further 14 days this patient showed a raised influenza-B-antibody titre. The course was similar, though less typical, in the second of these two cases.

Simultaneous rise in antibody titre to influenza-B and para-influenza 2 virus took place in one case, and to influenza-B and influenza-C virus in one case.

Influenza-B-virus infection was associated with bacterial infection in 6 cases. In four of these antibodies to virus and bacteria developed at about the same time. In two of the cases the bacteria were *H. influenzae*, in one *beta*-haemolytic streptococci, and in the third antibodies both to pneumococci and *beta*-haemolytic streptococci were involved. These four cases may probably be regarded as ordinary, complicated influenza-B virus infection.

The two others presented interesting combinations of virus and bacterial infection.

In one case the antistreptolysin titre was raised at the onset of the illness, further increasing during the subsequent course. The recruit was kept in the sick bay for 7 days, under penicillin therapy. Pyrexia was mild and symptoms were limited to moderately severe tonsillitis. The influenza-B-antibody titre remained normal until the 11th day, the rise in titre occurred between the 11th and 21st days.

In one case a slight tonsillitis was manifest. *H. influenzae* were demonstrated in the first nasal swabs, the 10th day after the onset of symptoms a significant increase in the serum complement-fixation titre to *H. influenzae* was noted, but no increase in the influenza-B virus antibody titre (an increase in the latter was noted after a further 13 days).

Infection with adenovirus type 3 and para influenza type 2 occurred simultaneously in one case. In addition three para influenza infections were demonstrated by serological tests, one of these being complicated by pneumococcal infection.

It is worthy of note in connexion with the virus aetiology, that adenovirus type 3 was isolated in no less than 10 cases, but that a significant rise in titre was recorded only in 5.

On the basis of the serological findings two cases were classified as ornithosis. One of these was characterized by slight catarrhal symptoms, the condition becoming complicated by otitis and bilateral conjunctivitis on the 12th day. Routine cultures yielded *Staph. aureus*, meningococcus, and adenovirus, but no rises were seen in these antibody titres. In the second case clinical signs of pneumonia were demonstrable for the first time. The patient was discharged after 14 days, and after a further 7 days developed sore throat, cough, ulcers and blisters on the hard palate.

Paul Bunnell's reaction was positive in one case. The course of the illness and the pharyngeal findings tallied with a diagnosis of mononucleosis.

One case of primary H influenza infection occurred. In 5 cases the causative organism remained unidentified.

During the autumn of 1959 7 cases were seen which were classed as ARD, one of these being ascribable to adenovirus infection, one to beta haemolytic streptococci. The others remained undiagnosed.

During the winter of 1960 a total of 39 cases occurred and as had been the case during the preceding winter influenza predominated, this time influenza A.

12 cases of uncomplicated influenza A infection occurred, and 11 cases in which influenza was accompanied by one or two other infections. Influenza was considered to be the primary infection in 6 of these, one of which was a double infection with adenovirus. Two of these were complicated by beta haemolytic streptococcal infection and three by pneumococcal infection.

The three remaining cases showed both influenza A and adenovirus infection, the influenza-virus infection probably having developed later than the infection due to the adenovirus. In one of these three cases the clinical picture was similar to the one known from adenovirus infection, the symptoms including ulcers of the hard palate, although antibodies to adenovirus and influenza A appeared at about the same time. In two cases the course of development of antibodies suggested that the adenovirus infection had preceded the influenza. Adenovirus type 7 was isolated from one of these cases. The essential symptoms were pyrexia, malaise, coryza and hoarseness. The patient was treated in the sick bay for 9 days. Raised adenovirus antibody titre was demonstrated on the 12th day, in the absence of antibodies to influenza virus type A. On the 17th day the patient was readmitted to the sick bay, because of re-

TABLE
Symptoms and Signs of Acute Respiratory Diseases

Type of Infection	Number of cases	Signs								
		1	2	3	4	5	6	7	8	9
Adenovirus	6	6	2	5	3	—	5	—	—	1
Influenza A	12	12	6	7	5	7	11	1	1	1
Influenza B	13	13	11	6	13	12	11	—	—	4

- 1 Fever
- 2 Glandular enlargement
- 3 Nasal discharge
- 4 Infection of the palate
- 5 Infection of the tonsillar region

- 6 Infection of the pharynx
- 7 Exudative tonsillitis
- 8 Conjunctivitis
- 9 Pulmonary signs

currence of the respiratory infection. Blood samples taken on the 42nd day of the illness showed titres of 1/160 and 1/320 for adenovirus and influenza virus, respectively.

Adenovirus infection not associated with influenza occurred in 7 cases, of which one showed raised antistreptolysin and antipneumolysin titres 24 hours after the onset of symptoms, whereas a raised anti-adenovirus titre was not obtained until the 8th day. Adenovirus type 7 was isolated in 6 of the cases, and a raised antibody titre was recorded only in one case.

Primary bacterial infection occurred in 5 cases. In 2 cases, the causative agent was beta-haemolytic streptococci in one case *Staph. aureus*, pneumococci in one case, and beta-haemolytic streptococci + *Staph. aureus* in one case.

The causative organism remained unidentified in 6 cases.

Thus uncomplicated infection with influenza types A and B and adenovirus occurred in 12, 13 and 6 cases, respectively, in these groups, incidence being higher here than in any other group. The symptoms occurring in these selected, aetiological "pure" infections, are illustrated in Table 1.

Because of the small number of cases recorded in this table a certain caution must be exercised when conclusions are drawn. It is evident, however, that an adenovirus infection may produce symptoms identical with those of influenza, namely, pyrexia, malaise, headache, cough, and hoarseness. One feature, not seen in the adenovirus infections in this series, is the non-exudative tonsillitis common in influenza.

Minor Respiratory Illness

This group is illustrated in Fig. 4 and comprises 73 cases. The most outstanding feature is the comparatively high number of cases, 33, in which the aetiology could not be established.

During the winter of 1959, 32 cases occurred. Influenza B was represented by 10 cases, all of which were uncomplicated. Para-influenza was diagnosed in 6 cases, one of which was complicated by pneumo-

Caused by Adenovirus of Influenza A and B Virus

Symptoms												
10	11	12	13	14	15	16	17	18	19	20	21	22
6	4	1	11	2	—	3	3	11	11	2		1
12	7	2	10	3	—	6	11	4	8	2		1
11	6	4	6	—	2	5	8	3	4	3	—	2
10 Malaise					15 Sore eyes					20 Nausea		
11 Headache					16 Sore throat					21 Diarrhoea		
12 Myalgia and Arthralgi					17 Dry cough					22 Chills		
13 Coryza					18 Productive cough							
14 Sneezing					19 Hoarseness							

coccal infection Adenovirus infection occurred in 3 cases, in one of these combined with para-influenza type 2. Noteworthy, too, was the exceptional case in the series presenting primary influenza type C and para-influenza type 1. Primary bacterial infection, a double infection with beta-haemolytic streptococci and pneumococci, occurred in only one case. The aetiology remained obscure in 12 cases.

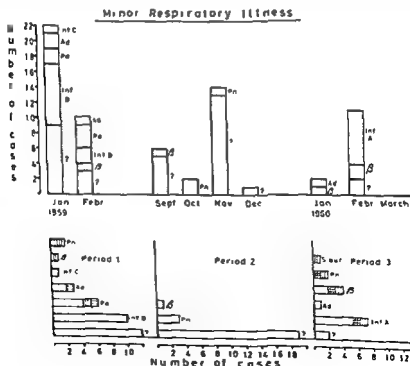


Fig. 3

Distribution of cases on the different months of the year, correlated to aetiological confirmed infections. For abbreviations see Fig. 1

During the autumn of 1959, 23 cases occurred, but the causative organisms were identified in only 4 of these. Three were due to pneumococci and one to beta-haemolytic streptococci.

During the winter of 1960, 13 cases referable to this group were noted. Influenza-A infection was present in 6 cases; 4 of these were uncomplicated. One case of influenza was complicated by concomitant pneumococcal infection, and influenza A was diagnosed concomitant with pneumococci and beta-haemolytic streptococci in one case. In the latter case streptococci were isolated from the first throat swab, and an increase in the antistreptolysin and also in antipneumolysin titres was seen as early as on the 13th day of illness at which time the anti-influenza-A titre was only insignificantly raised. On the 20th day the anti-streptolysin titre had fallen, and the influenza titre risen from 1/10 to 1/320. Apparently this case may have been a primary bacterial infection followed by influenza A, the latter being nosocomial.

One adenovirus type 7 infection and 3 primary bacterial infections also occurred, two being due to beta-haemolytic streptococci and one to beta-haemolytic streptococci + *Staphylococcus aureus*. The aetiology remained obscure in 2 cases.

Distribution of Primary Infections on the four Groups

The most common infections in this series are influenza A and B, adenovirus infection due to types 3 and 7, and infection caused by beta-haemolytic streptococci. Table 2 illustrates the distribution on the four groups of primary infections caused by the above pathogens.

Aetiologically "pure" cases only are included in table 2, and hence the number of cases is too small to allow for definite conclusions to be drawn. It is found, however, that influenza A and B and adenovirus infections are most common in the group acute respiratory diseases. Mild influenza-B infections are also represented. Most of the beta-haemolytic streptococcal infections are to be found in the group exudative pharyngitis. The group minor respiratory diseases includes the highest number of cases in which the causative organism could not be

TABLE 2
Comparison between Microbiological Diagnoses and Clinical Syndromes of Respiratory Diseases

Microbiological diagnosis	Exudative pharyngitis	Thrush, conjunctivitis	Acute respiratory disease	Minor respiratory disease
Influenza A	1	3	12	4
Influenza B	1	7	11	10
Adenovirus	1	1		1
Beta haemolytic streptococci	9	2	1	1
Diagnosis unknown	5	0	10	11
Total number	19	19	51	53

identified although many such cases may be found also in the group acute respiratory diseases

DISCUSSION

The four groups of diseases discussed in this article have probably never been regarded as disease entities as regards aetiology. It has been attempted however to analyse the symptoms of these virus and bacterial infections in order to detect if possible features characteristic of the several micro-organisms. Schultz and collaborators (7) who examined recruits claim to have found a definite difference between for example influenza A2 and adenovirus 4 infection the former being characterized by typical symptoms of influenza *vi* acute onset pyrexia headache malaise sore throat and a dry cough the latter being associated with a more gradual onset sore throat malaise (representing the main feature) hoarseness coryza and headache.

A similar analysis illustrated in Table 1 discloses that the pathological pictures of influenza and adenovirus infection are rather similar differences in the present series being so slight as to make it impossible to arrange the clinical entities according to "pure" cases.

Concerning the complicated cases it is noted that influenza A produced exudative pharyngitis in 5 cases of which only one was uncomplicated the others showing concomitant infection with beta haemolytic streptococci. In one of these the influenza appears to be secondary to the bacterial infection. Only three pure cases of influenza A are classed as pharyngoconjunctivitis. In the other cases concomitant adenovirus or bacterial infection were noted.

Most of the pure cases of influenza A (11-12 cases) are to be found in the ARD group which also includes 7 cases of influenza A with concomitant adenovirus infection together with a few cases complicated by bacterial infection however. Influenza A also occurs in the MRI group (1 uncomplicated cases and 2 with concomitant bacterial infection).

With regard to influenza B the state of affairs is similar although this type is more common than influenza A in the MRI group.

Concerning influenza occurring during "between" years it may be said in general that the clinical picture is highly dependent on the state of immunity of the population and on the occurrence of concomitant virus and bacterial infection and hence is strikingly heterogeneous. This observation has already been mentioned but it does not tally exactly with the results reported by Schultz and his collaborators. However the American investigations were carried out in connection with the first influenza A2 infections in U.S.A. Primary infection with a new mutant may possibly produce a more uniform clinical picture in a population than would be the case if it appeared during a "between" year.

The number of cases of adenovirus infection is too small to allow for conclusions to be drawn. The present investigations confirm the original observation by *Hilleman* and collaborators (4), that this virus appears concomitant with influenza A. In our series this is also true of influenza B. Whether or not influenza and adenovirus appear independently of one another during the "common-cold season" or if they have some symbiotic relationship cannot of course be determined on the basis of this small series. The adenovirus cases seem to be distributed on the four groups in much the same manner as the influenza cases, as far as can be judged from the available material.

Diseases due to primary beta-haemolytic streptococcal infections are to be found chiefly among the cases of exudative pharyngitis, and are represented by no less than 9 "pure" cases, beta-haemolytic streptococci being isolated from the throat in all of these. This organism was fairly common also in the ARD group here including 8 cases, of which two only were "pure". The remainder were distributed on the other two groups.

In the present series a significant rise in titre during the course of the illness has been regarded as aetiologically significant. On the basis of this norm the series shows an incidence of virus infections of altogether 52.1 per cent, and of bacterial infections without a well established, concomitant virus infection 14.0 per cent. Aetiological confirmation was thus obtained in 66.1 per cent of cases. As stated in Article 2, complement fixation tests against 11 influenzae were not carried out in the series originating from the autumn of 1959 and the winter of 1960. This probably has no important effect upon the results on the whole. Out of the total number of infections 36.7 per cent were uncomplicated virus infections. Concomitant virus or bacterial infections was present in the remaining cases. Of the total number of infections 9.7 per cent were "pure" bacterial infections (mostly beta-haemolytic streptococci). It must be stressed, however, that these confirmed diagnoses do not exclude the possibility that other, not diagnosed, virus infections may have occurred in the series. This is particularly true of periods during which the numbers of "negative" cases were especially high, but also in cases in which some other causative organism had been identified. Indeed an apparently primary bacterial infection may be secondary to an undiagnosed virus infection. Additional virus infections may have been present concomitant with demonstrated influenza and adenovirus infections without having been diagnosed.

The incidence of positive cases differed during different periods, being high during the two influenza epidemics in the winters of 1959 and 1960. It was high also during the outbreak of the beta-haemolytic streptococcal infection in September 1959, but was strikingly low in November 1959. This implies that in outbreaks of "conventional" types of infection the cases can apparently be mapped out satisfactorily but that outbreaks may occur in which special attention should be paid to the establishment of the true diagnosis.

A striking difference was noted between the types of infection occurring during autumn and winter. Indeed in early autumn the incidence of adenovirus infections might increase, but bacterial infections would predominate. Both winters were dominated by virus infections. The investigations carried out during the autumn of 1960 have disclosed that infections were of the same type as those seen during the autumn of 1959. An account of these recent results will appear in a future paper, and studies will have to be continual for several years before any definite conclusions can be drawn.

It is interesting to note that cases of primary beta haemolytic-streptococcal infection followed by influenza virus infection, possibly of a nosocomial nature occurred. A few cases were seen in which penicillin treatment of the primary infection resulted in subsequent infection with *H. influenzae*. No serious nosocomial infections occurred, however.

SUMMARY

Studies on the acute respiratory infections occurring among military personnel during three periods during the years 1959-1960, viz. the winter of 1959, the autumn of 1959, and the winter of 1960, involved a detailed investigation of 208 cases. The incidence of illness was low throughout. Two "between" year epidemics (influenza A2 and B, respectively) occurred. Attempts to correlate the clinical and aetiological findings have not given very encouraging results. 'Typical' cases, showing the accepted clinical picture of influenza and beta haemolytic-streptococcal infection, were encountered, but in most cases the clinical picture upon which the clinical diagnosis was based showed that several microbiological agents were included in the etiology.

The causative organisms were identified in about 66 per cent of the cases. Of these 52.1 per cent were cases of virus infection, and 14.0 per cent bacterial infection in which a primary virus infection was not demonstrable. The seasonal distribution of the various infections is discussed.

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YEASTS OF HUMAN ORIGIN

By

A. STENDERUP and G. THOMSEN PEDERSEN

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The purpose of the present paper is to give a report on the species of yeasts in, and the frequency of isolation of the species from, various human materials submitted for bacteriological and mycological examination during the two-year-period 1959-1960.

MATERIALS AND METHODS

Apart from nasal swabs, most of which were obtained from healthy individuals, the specimens originated from patients, mainly from Aarhus Municipal Hospital, but specimens were received also from many hospitals in other parts of the country. In some cases the specimens were submitted because a mycotic infection had been suspected, but in the present study it has not been attempted to correlate occurrences of fungi with occurrences of clinical mycosis.

Cultivation of yeasts was performed by seeding on Sabouraud glucose agar, Littman's ox bile agar and on brain heart infusion agar with 10 per cent blood. Antibiotics were added to the last two media in order to inhibit bacterial growth. In addition, all samples were seeded in glucose-peptone solution. The samples were incubated at 25 and 37° C and studied for growth at regular intervals for 3 to 4 weeks. Isolated yeasts were transferred to malt-agar plates.

The classification introduced by Lodder & Kreger van Rij (1952) was used, except in species of the genus *Hansenula* which were classified according to Wickström (1951).

RESULTS

Table 1 shows all of the yeasts classified during the period together with the sources from which they were derived. In some cases, we received the fungi in pure cultures, in others, they originated from samples on which mycological studies only were performed.

Material from the bronchi was collected during bronchoscopy. Usually the urine samples studied would be catheter urine from women and voided urine from men. The swabs from the ears mainly originated from acute or chronic cases of otitis media. Wound samples consisted of secretions from wounds, fistulae, etc. Materials from the kidneys, liver, gastro-intestinal tract and lungs were obtained either at operation or autopsy. Under the heading Miscellaneous are listed all samples of unknown origin.

A total of 968 yeast cultures were obtained. The majority of these belonged to the genus *Candida*, in which *C. albicans* was the predominant species, being represented by 659 isolates, i.e. 68 per cent. This figure is somewhat higher than the one reported by Mackenzie (1961), who recently has published a similar study from Scotland. *C. krusei*, *C. tropicalis*, *C. pseudotropicalis*, *C. zeylanoides* and *C. parapsilosis* each represented about 2 per cent of the isolated strains, while the remaining *Candida* species were even rarer.

The most common genus but one was *Torulopsis*, the most frequent member here of being *T. glabrata*, which was present in 137 cases, i.e. in 14 per cent of all of the isolated strains, the occurrence of other *Torulopsis* species was rare. In a few isolates belonging to this genus the species could not be unquestionably determined.

Of the members of the genus *Rhodotorula* we isolated only *R. mucilaginosa*, viz. in about 2 per cent.

Species of the genera *Cryptococcus* and *Trichosporon* were rarely isolated.

Among the sporogenic yeasts, *Saccharomycetaceae*, *Saccharomyces cerevisiae* was the one most commonly encountered.

Table 2 gives a survey on all of the samples in which simultaneous bacteriological and mycological studies were performed and thus shows how often and from which sources yeasts assumedly may be isolated in samples submitted for bacteriological examination.

It is seen that the frequency with which yeasts were isolated from the oral cavity was very high (71 per cent). These samples include swabs from the pharynx, tonsils, gingiva, and tongue together with sputum specimens, since indeed it must be assumed that yeasts isolated from sputum generally originate from the oral cavity. Yeasts were isolated from the bronchi in 22 per cent, from urine in 8 per cent, from ears in 4 per cent, from wound secretions in 7 per cent and from the nose in 3 per cent. It is seen also that the two species most frequently encountered are *Candida albicans* and *Torulopsis glabrata*, together these two represent about 87 per cent of all species isolated from the oral cavity. Other species of the genus *Candida* (apart from *C. albicans*) were, as a whole, isolated more rarely than *Torulopsis glabrata*. The incidence of the other genera was very low. The family of *Saccharomycetaceae* was found only in 1.6 per cent.

DISCUSSION

The frequency with which yeasts were isolated from the oral cavity is in agreement with figures reported by Helms (1956) and Mackenzie (1961).

Yeasts from urine were found also with the same frequency as the one reported by Mackenzie (1961). However, this author found *Torulopsis glabrata* more frequently than *Candida albicans*, which has not

TABLE
Sources

	Oral cavity	Oeso- phagus	Inte- stines	Lac- tes	Tra- chea	Bron- chi	Lung	Kid- ney
1 SACCHAROMYCETACEAE								
<i>Genus Saccharomyces</i>								
<i>S. cerevisiae</i> Hansen	5	1	-	5		1		
<i>S. cerevisiae</i> Hansen var ellipsoideus (Hansen) Dekkcr	1	-					1	1
<i>S. fragilis</i> Jørgensen	1	-	-					
<i>S. roiseri</i> (Guilliermond) nov. comb.		-				1		
<i>Genus Pichia</i>								
<i>P. membranaefaciens</i> Hansen	1			-		1		
<i>P. fermentans</i> Lodder	1		-					
<i>Genus Hansenula</i>								
<i>H. californica</i> (Lodder) Wickerham	1			1				
<i>Genus Debaryomyces</i>								
<i>D. hansenii</i> (Opf.) nov. comb.	1							
2 CRYPTOCOCCACEAE								
<i>Genus Cryptococcus</i>								
<i>Cryptococcus</i> species	2							
<i>Genus Torulopsis</i>								
<i>T. glabrata</i> (Anders. n.) Lodder et de Vries	55	6	2	11	4	11	11	3
<i>T. candida</i> (Saito) Lodder	1					1		
<i>T. famata</i> (Harrison) nov. comb.						1		
<i>T. gropengiesseri</i> (Harrison) Lodder	1							
<i>T. stellata</i> (Kromer et Krumholz) Lodder	1							
<i>T. wake</i> (Saito et Ota) nov. spec.	1			1				
<i>T. inconspicua</i> nov. spec.								
<i>Torulopsis</i> species	2	1				1		
<i>Genus Candida</i>								
<i>C. albicans</i> (Robin) Berkhout	342	20	8	23	13	79	16	7

1

Sources

Urine	Vagina	Ear	Abscess	Peritoneum	Wounds etc	Nose	Nasal muzzes	Pleural cavity	Liver	Miscellaneous	Total
-	5	-	-	-	-	-	-	-	-	3	20
-	-	-	-	-	-	-	-	-	-	-	3
-	-	-	-	-	-	-	-	-	-	-	1
-	-	-	-	-	-	-	-	-	-	-	1
-	-	-	-	-	-	-	-	-	-	-	2
-	-	-	-	-	-	-	-	-	-	-	1
-	-	-	-	-	-	-	-	-	-	-	3
-	-	-	-	-	-	-	-	-	-	-	1
-	-	-	-	-	-	-	-	-	-	-	2
13	8	2	-	1	3	-	-	1	-	6	137
-	-	-	-	-	-	-	-	-	-	-	2
-	-	-	-	-	-	-	-	-	-	-	2
-	-	-	-	-	-	-	-	-	-	-	1
-	-	-	-	-	-	-	-	-	-	-	1
-	-	-	-	-	-	-	-	-	-	-	2
-	-	-	-	-	-	1	-	-	-	1	2
-	-	-	-	-	-	-	-	-	-	-	4
26	20	18	2	5	14	4	1	0	3	32	659

TABLE 1

	Oral cavity	Esophagus	Intestines	Farces	Trachea	Bronchi	Lungs	Kidney
<i>C. mycotherma</i> (Hess) nov. comb.	1					1		
<i>C. krusseti</i> (Cast) Berkhout	5	2				5		1
<i>C. tropicalis</i> (Cast) Berkhout	10	1				1	1	1
<i>C. pseudotropicalis</i> (Cast) Basgal	1	1				2		
<i>C. guilliermondii</i> (Cast) Langeron et Guerra								
<i>C. cytharoides</i> (Cast) Langeron et Guerra	5		1	1	1	1		
<i>C. parapsilosis</i> (Ashf) Langeron et Talice	1		2	2		1	2	
<i>C. parapsilosis</i> (Ashf) Langeron et Talice var <i>intermedia</i> van Rij et Verona						2		
<i>C. robusta</i> Diddens et Lodder								
Genus <i>Trichosporium</i>								
<i>T. cutaneum</i> (de Barm Gougerot et Vaucher) Ota				1			1	
<i>T. capitatum</i> Diddens et Lodder	1							
Genus <i>Rhodotorula</i>								
<i>R. mucilaginosa</i> (Jor- gensen) Harrison	1	1	3	1		2	5	1
Unidentified	8			1		2	1	

been the case in the present study in both series however figures are rather small. In view of this high frequency (8 per cent) a striking feature is that yeasts only infrequently are seen to represent the causative agent in urinary tract infections. Microorganisms from the urethra and the urethral environment are readily admitted to urine secured for bacteriological examination. Using a very careful technique Guze & Haley (1958) isolated yeasts in only 15 of 1500 urine specimens i.e. 1 per cent.

Yeasts were found in 22 per cent of the studied specimens of bronchial secretions. This figure is slightly higher than the one reported in a previous study (Buhl & Stenderup 1958). In the aspiration of bronchial secretion special precautions should be taken to prevent secretion from the nasopharynx to be carried down into the bronchi. As we do

TABLE 2
Yeast Isolated from Bacteriological Specimens in the Years 1959-1960

	No of sample examined	Yield in Yeasts		Candida Albicans		Torul glabrata		Other Candida species		Sacchar
		No	%	No	%	No	%	No	%	
al cavity	625	442	71	330	53	53	8	33	5	10
onchi	506	113	22	79	16	11	2	13	2	3
rine	524	44	8	23	4.4	13	2	2	0.4	
ir	685	26	4	18	3	2	0.3	6	1	
ounds etc	316	22	7	14	0.5	3	0.1	3	0.1	
osq	184	5	3	4	2					
asal sinuses	226	2								
iscellaneous	455	68	15	51	12	6		6		2

be of significance in the increased frequency of *Candida* infections (Brattlund & Holten 1954, Torack 1957)

It is often stated that *C. albicans* is the only species of this genus capable of causing infection in man. However, other *Candida* species may also give rise to infection, we have seen a case recently in which infection unquestionably was caused by *C. parapsilosis*. The patient was a boy, aged 16, in whom this species was isolated repeatedly from the blood and cerebroventricular fluid (Marcussen & Stenderup).

Like Benham (1957), we isolated most frequently *C. krusei*, *C. tropicalis*, *C. pseudotropicalis* and *C. parapsilosis*, these as well as the other species of the genus *Candida* must be considered potentially pathogenic. The species of *Candida* which relatively rarely are isolated from human sources are fairly common in nature (Lund 1954, 1958), accordingly, it can not surprise that they occasionally may be isolated also from human beings.

Torulopsis glabrata, representing 14 per cent of the yeasts isolated in this study, has received little attention in the medico mycological literature. It is seen rarely only and is hardly included in the normal microflora. Originally it was isolated from human faeces (Anderson 1917). It was found in the respiratory tract by Reutersol (1953) and Dietrichson (1954), and mentioned by Ruddell & Clayton (1958), it was demonstrated in urine by Iodder & de Vries (1937) and Guze & Haley (1958). *T. glabrata* in the external genitalia was first isolated from a vulval ulcer by Carol (1935) and later from vaginal secretions by Petrá & Vojtěchovská (1956).

T. glabrata is found in nature. For instance, it has been observed in shrimps from the Gulf of Mexico (Pfaff, Wraak & Williams 1952) and in orange juice (Recca & Wraak 1952). Ainsworth & Austwick (1955) isolated it from pigs and poultry.

However, it should be mentioned that Lund (1954), in studies on the occurrence of yeasts in nature in Denmark, isolated species of the genus *Torulopsis*, but not of *T. glabrata*.

The literature on the ability of *T. glabrata* to cause infections is very sparse. Wickerham (1957), who incidentally expressed the view that *T. glabrata* is recovered now from human sources more frequently than before, has isolated this organism repeatedly from the blood of a patient who no doubt suffered from a yeast mycosis, although the case history is not reported in detail. Black & Fisher (1937) suggested that *T. glabrata* was of a pathogenic significance in a pulmonary infection in a 10 year-old boy but this case must be considered to be doubtful.

The ability of *T. glabrata* to produce infections in animals was studied by Benham (1955) in rabbits where the species was found to be apathogenic. Iodder & de Vries (1937) and de Vinjer (1941) studied this problem in rats and mice, respectively, but found no definite pathogenicity. Black & Fisher (1937) inoculated *T. glabrata* into rats, hard nodules developed in the omentum, from which the organism

TABLE II
Yeast Isolated from Bacteriological Specimens in the Years 1959-1960

	No of sample examined	Yielding Yeasts		Candida Albicans		Torul glabrata		Other Candida species		Saccharomycetaceae	
		No	%	No	%	No	%	No	%	No	%
Oral cavity	625	442	71	330	53	53	8	33	5	10	1.6
Bronchi	506	179	22	79	16	11	2	13	2	3	
Urine	524	44	8	23	4.4	13	2	2	0.4		
Ear	685	26	4	18	3	2	0.3	6	1		
Wounds etc	316	22	7	14	0.5	3	0.1	3	0.1		
Nose	184	5	3	4							
Nasal sinuses	226	2									
Miscellaneous	455	68	15	51	12	6		6		2	

In a series of 321 specimens on which simultaneous bacteriological and mycological studies were performed yeasts were present in 722 i.e. about 21 per cent. The frequency with which yeasts were isolated from the various regions of the human body varied widely, from 71 per cent in the oral cavity to 22 per cent in the bronchi, while they were more rare in the urine, the ears, nose, and in wounds.

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could be cultivated. After intravenous and intraperitoneal injections of *T. glabrata* into albino mice, *Lopez-Fernandez* (1953) found yeast cells embedded in macrophages and stated that the histological picture resembled the one of histoplasmosis.

Thus, results obtained in animal experiments are contradictory.

Rhodotorula mucilaginosa, which has been recovered relatively often, was isolated for the first time in Copenhagen in 1909 by *Alfred Jorgensen*. We do not know of cases in which this organism has excited illness in man. It is very common in the atmosphere, which explains that it is often isolated from human sources.

Trichosporon cutaneum, which we found on a few occasions, has previously been isolated from human sources. Thus, *Lodder & Kregger-van Rij* (1952) reported that 18 of the 28 strains they studied were of human origin. *Reiersol* (1955) stated that *T. cutaneum* may give rise to otomycosis.

The ascospore-forming yeasts, *Saccharomycetaceae*, are isolated occasionally from human sources (e.g., *Mackenzie* 1961). The most common species is *Saccharomyces cerevisiae*. Probably the explanation of its presence in the human organism is that contact may easily be established with it through baker's yeast.

As already mentioned, the increase in fungus infections has been attributed partially to the use of antibacterial agents. One of the reasons for this allegation is that these drugs alter the composition of the normal micro-flora, because bacteria which are sensitive to antibiotics are more or less eliminated, thus providing possibilities for an increased proliferation of the fungi.

As antimycotic agents gain ground in modern therapy, they will give rise also to changes in the micro-flora. Hence it is still justified to carry out investigations by which to follow such changes, especially because many of the species of yeasts which we so far have considered to be apathogenic ultimately may prove to be capable of producing infection in the presence of favourable conditions. Moreover fungus infections occur frequently as complications in many diseases, and it may be presumed that infections which at present are of unknown aetiology eventually may prove to be referable to fungi.

SUMMARY

The results of a classification of yeasts recovered from human sources during a two-year period are reported. A total of 969 yeast cultures were obtained. The yeast most commonly isolated was *Candida albicans*, which represented 68 per cent, the second being *Torulopsis glabrata* representing 14 per cent. *C. krusei*, *C. tropicalis*, *C. parapsilosis*, *Rhodotorula mucilaginosa* and *Saccharomyces cerevisiae* each represented about 2 per cent of the species recovered, while the other species were more rare.

EINE VEREINFACHUNG DER SEROLOGISCHEN ARIZONA-DIAGNOSE

von

F. KAUFFMANN UND R. ROHDE

Eingegangen 3 XII 61

In einer vorhergehenden Mitteilung von F. Kauffmann (1) war das genus *Salmonella* in 2 biochemisch definierte subgenera eingeteilt worden. Gleichzeitig wurde darauf hingewiesen, dass die Arizona-Gruppe als *Salmonella* sub-genus III bezeichnet werden könne, da Dulcitate negative *Salmonella* species sowohl im sub-genus I als auch im sub-genus II vorkommen. Ferner wurde gesagt, dass die serologische Bestimmung von Arizona-kulturen mit Hilfe des Kauffmann-White-Schemas (K-W-Schema), das für diesen Zweck erweitert oder vereinfacht werden könne, erfolgen kann.

In der folgenden Darstellung schlagen wir nun vor, die serologische Arizona-Diagnose mit Hilfe des vereinfachten K-W-Schemas vorzunehmen und die Arizona-Gruppe in klassifikatorischer Hinsicht als sub-genus III des genus *Salmonella* zu betrachten.

Wir unterscheiden also im genus *Salmonella* 3 biochemisch definierte subgenera:

- Sub-genus I oder typische *Salmonella*-Bakterien,
- sub-genus II oder atypische *Salmonella* Bakterien und
- sub-genus III oder Arizona-Bakterien = *Salmonella* arizonae

Betreffs der biochemischen Differentialdiagnose sei auf das Buch von F. Kauffmann (2) „Die Bakteriologie der *Salmonella* Species“ verwiesen.

Diese 3 subgenera werden mit Hilfe der Serologie in species = Gruppen verschiedener sero-fermentativer Phag-Typen eingeteilt und können in serologischer Hinsicht alle mit Hilfe des K-W-Schemas diagnostiziert werden.

Die type species des sub-genus I ist *Salmonella cholerae-suis*, die type species des sub-genus II ist *Salmonella dysenteriae*, während die type species des sub-genus III die Arizona species 1,2, 1,2,5 (= 51, 21) ist.

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Obwohl es also möglich ist, die *Arizona species* in das originale K W Schema einzufügen, so wollen wir dieses nicht tun, sondern schlagen vor, ein besonderes *Arizona* Schema entsprechend dem vereinfachten K W Schema aufzustellen. In diesem Schema werden so weit wie möglich die Symbole des vereinfachten K W Schemas benutzt und nur in denjenigen Fällen, in denen noch keine Antigengemeinschaften zwischen *Arizona* und *Salmonella* nachgewiesen sind, werden bis auf weiteres *Arizona* Symbole angewandt.

TABELLE 2
Antigenstruktur von *Arizona*-Species

kultur	Nach Ivaris			Nach Kauffmann & Floide		
	O	H		O	H	
		1. Flase	2. Flase		1. Flase	2. Flase
DC 5	12	12.5		51	24	
41 a-57	13	12.10		44	24	
CD41 44	14	12.5		53	24	
148	1.33	26	31	58	Ar 26	z
1840-54	5	17.20		48	2.5	
So 50	6	13.14		Ar 6	G	
2432 53	7a,b	12.6		18	24	
1995 58	7a,c	27	18	C-18	2.5	Ar 38
(DA) 426	8	17.8		Ar 8	24	
499	9a,b	13.15		50	G	
3600 56	Ja,c	33	31	50	1	z
36608	10a,b	12.6		40	24	
396-56	10a,c	13.15		40	G	
Ore 181	11	16.17.18		45	2.5	
142 56	11	35	28	45	a	e n
51 21 x 74	12	1.6.7		17	24	
52 4	13	13.14		41	G	
1 c 123	14	1.6.7.9		56	24	
Pe 139	15	13.14		42	G	
2 88 55	16	24	31	38	r	z
594 54	16	23	14	38	L	Ar 34
2224 51	16	22	37	38	k	Ar 37
1158 58	16	39	25	38	Ar 37	Ar 23
Min 78	17	23	25	11	k	Ar 25
38 8 58	18	13.11		13	24	
320 51	19	12.6		Ar 17	24	
4 87 7	20	23	30	35	1	1
1450 53	21	12.6		43	24	
2953 55	22	12.5.6		21	24	
1335 52	23	33	25	47	1	Ar 25
256 58	24	22	25	Ar 24	k	Ar 25
1 c 110	25	27	28	16	21	e n
261 9	26	32	21	Ar 26	r	24
1 ex 31 11	27	23	25	6	1	Ar 25
3592 56	28	23	25	47	L	Ar 25
2307 54	29	33	40	Ar 29	1	Ar 40
46 11 54	30	33	25	52	L	Ar 25
3543 54	31	23		6	24	e n
16 5 55	32	12.6		57	1	
195	34	33	28			

Die Antigen Formel dieser *type species* des originalen *Arizona* Stammes war von F. Kauffmann (3) mit 33 z1 z3 z7 angegeben worden. Die O Gruppe 33 wurde aber aus dem K W Schema ausgeschlossen als eine selbständige *Arizona* Gruppe von Edwards, West & Bruner aufgestellt wurde. Die heutige *Salmonella* O Gruppe 51 ist mit der früheren O Gruppe 33 identisch. Die früher mit z1 z3 z7 angegebenen H Antigene (= 125) des originalen *Arizona* Stammes werden im vereinfachten K W Schema und damit auch im vereinfachten *Arizona* Schema mit z1 bezeichnet.

Betreffs der Serologie der *Arizona* Gruppe sei auf die letzte zusammenfassende Arbeit von Edwards, Tife & Ramsey sowie auf die dort angegebene Literatur verwiesen.

TABELLE 1
Antigengruppenbezeichnungen *Salmonella* und *Arizona*

H Antigen				O Antigen			
				11a		211c	
Arizona	51	Arizona	51	Arz	51	Arz	51
12	51	21	33	195	z4	21	z3
13	44	22	21	196	z4	22	
14	53	23	47	1956	z4	28	cn
13J	58	24		1910	z4	30	1
5	48	25	16	1236	z4	31	z
6		26		1311	z4	34	
7a b	18	27	6	167	z4	37	
7a c	C+18	28	47	167J	z4	38	
8		29		178	z4	40	
Ja b	0	30		1314	G		
Ja c	50	31	5	1315	G		
10a b	40	32	6	16718	z		
10a c	40	34	57	170	z3		
11	45				k		
12	17			3	l		
13	41			24	r		
14	56			6			
15	42			7	z1		
16	38			29	l		
17	11			32	c		
18	13			33	l		
19				3	d		
20	35			33			

Die neuen Antigen Beziehungen zwischen *Salmonella* und *Arizona* Kulturen waren von Anfang an bekannt und sind in der Arbeit von Edwards, Tife & Ramsey zusammengestellt. Inzwischen sind weitere Antigen Beziehungen festgestellt worden, sodass man sagen kann, dass die meisten *Arizona* O und H Antigene im K W Schema vorhanden sind und deshalb mit *Salmonella* Antigenformeln wiedergegeben werden können.

Das Symbol L umfasst alle Phasen mit den 1,1 - 1,w - 1,z₁₃ - 1,z₁₃,z₂₃ - 1,z₂₃-Antigenen, während das Symbol l alle "unspezifischen" Phasen wie 1,2 - 1,3 - 1,6 - 1,7 - 1,5,7 und z₆ umfasst. In der entsprechenden Weise umschließt das Symbol G alle Phasen mit dem g Komplex, doch sind bisher bei *Arizona*-Kulturen vorwiegend die Antigene g, (p) gefunden worden.

Bei den O-Antigenen bedeutet das Symbol C ein polyvalentes Antigen, das den species *S. thompson*, *S. newport*, *S. carrau* und *S. onderstepoort* entspricht, also die O-Faktoren 6,7,8,14,24 und 25 umfasst. Alle übrigen Zahlen entsprechen dem K-W-Schema.

In der Tabelle 2 ist die Antigenstruktur von *Arizona species* im Vergleich zum Antigenchema von *Edwards* und Mitarbeitern angegeben. In der Tabelle 3 ist die vereinfachte Antigenstruktur, dem vereinfachten K-W-Schema entsprechend, dargestellt.

DISKUSSION

Die serologische Diagnose der einzelnen *Arizona-species* wird heute - abgesehen von dem Laboratorium von P R Edwards in Atlanta - nicht routinemässig ausgeführt, weil man nicht über die nötigen Seren verfügt. Deshalb ist es im Interesse der praktischen Diagnose nötig, ein vereinfachtes Antigenchema aufzustellen und hierzu, soweit wie möglich, die bereits vorhandenen *Salmonella*-Seren zu benutzen.

Wie es aus den hier angegebenen Tabellen hervorgeht, können die meisten *Arizona species* mit Hilfe unabsorbierter *Salmonella*-Seren diagnostiziert werden. Nur 8 *Arizona* O-Seren und 5 *Arizona* H-Seren sind heute erforderlich, um die bekannten *Arizona-species* zu diagnostizieren. Es ist aber damit zu rechnen, dass auch diese Antigene, speziell die O-Antigene, bald bei *Salmonella-species* der sub-genera I und II gefunden werden.

In derselben Weise, in der durch die Aufstellung des vereinfachten Kauffmann-White-Schemas nicht das originale K-W-Schema beeinflusst wurde, wird auch durch das vereinfachte *Arizona*-Schema nicht das originale Schema von Edwards und Mitarbeitern beeinträchtigt. Für praktische und speziell für epidemiologische Zwecke genügt das vereinfachte Schema völlig, da *Arizona*-kulturen als Erreger menschlicher Erkrankungen nur relativ selten auftreten. Sie kommen hauptsächlich bei Kaltblütern vor, sodass wir in epidemiologischer Hinsicht keine besondere eingehende Differenzierung nötig haben.

ZUSAMMENFASSUNG

Es wird eine Vereinfachung der serologischen *Arizona*-Diagnose mit Hilfe des vereinfachten Kauffmann-White-Schemas vorgeschlagen. Die *Arizona-species* werden als sub-genus III des genus *Salmonella* betrachtet und können mit *Salmonella arizonae* bezeichnet werden.

Betreffs des vereinfachten K-W-Schemas sei auf das Buch von F. Kauffmann (2) „Die Bakteriologie der *Salmonella-Species*“ und die auf Seite 126 dieses Buches angegebene Literatur verwiesen

Mit Rücksicht auf die bakteriologische Praxis halten wir es für völlig ausreichend, Arizona-Kulturen mit Hilfe des vereinfachten K-W-Schemas zu diagnostizieren, d. h. die Diagnose mit Hilfe der diagnostischen *Salmonella*-Serien zu stellen, abgesehen von einigen, wenigen Arizona-Serien. Es sei betont, dass das originale Arizona-Schema nach Edwards und Mitarbeitern seine volle Gültigkeit behält, und dass es jedem Untersucher freisteht, dieses Schema anzuwenden

TABELLE 3
Vereinfachte Antigenstruktur von *Arizona Species*

Körper Antigen		Geißel Antigene	
		1 Phase	2 Phase
C	47	a	1
C+18	48	c	en
11	50	Q	z
13	51	J	730
16	52		
17	53	k	Ar 25
18	57	L	Ar 34
21	58	r	Ar 37
		z4	Ar 38
35	Ar 6	z10	Ar 40
38	Ar 8	z20	
40	Ar 19	z30	
41	Ar 24		
42	Ar 26	Ar 26	
43	Ar 29	Ar 39	
44	Ar 30		
45			

Die mit Buchstaben oder Zahlen bezeichneten Antigene sind *Salmonella* Antigene. Nur die allein in der *Arizona* Gruppe vorkommenden Antigene sind mit Ar 6 etc. angegeben

Die Resultate unserer vergleichenden Untersuchungen an *Arizona*- und *Salmonella species* sind in der Tabelle 1 wiedergegeben. Sie bestätigen frühere Ergebnisse von Edwards u. a., doch entspricht das *Arizona* H-Antigen 23 = *Salmonella* Symbol L bei den H-Antigenen der 1. Phase dem H-Antigen 1, v. des K-W-Schemas und nicht dem H-Antigen 1, z. Ferner entspricht das *Arizona* H-Antigen 30 = *Salmonella*-Symbol 1 bei den H-Antigenen der 2. Phase dem H-Antigen 1, 5, 7 des K-W-Schemas und nicht dem H-Antigen 1, 5. Diese beiden Differenzen beruhen darauf, dass von Edwards andere H-Faktor-Sera als von uns angewandt wurden. Im vereinfachten K-W-Schema und im vereinfachten *Arizona*-Schema spielen diese Unterschiede keine Rolle, da die vereinfachten Formeln mit L resp. 1 angegeben sind.

BRIEF REPORT

BILE ACIDS AS A CAUSE OF LIVER INJURY CIRRHOTIC EFFECT OF CHENODESOMOXYCHOLIC ACID IN RABBITS

By Paul Holsti

Following the observation that the administration of hog bile induces cirrhosis of the liver in rabbits (1, 2, 3), and the linking of this effect to certain bile acids of hog bile (4) extensive investigations were undertaken of the effect of various steroids related to bile acids.

Most of the investigated bile acids caused only insignificant alterations in the

It was further shown that conjugation with glycine did not abolish the cirrhotic effect of lithocholic acid. The keto derivative 3-mono-hydroxy-6-ketoallocholic acid at the tested concentration (0.5 per cent) was found to be ineffective. Investigations are underway in order to elucidate the structure of the active principle.

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BRICE REPORT

ABO BLOOD GROUPS IN ALCOHOLICS

By Erik Juel

The relationship of the ABO blood groups to disease has been the subject of many papers the last years (1) and there is now an overwhelming evidence for several associations especially concerning diseases in the gastro intestinal tract. As nothing can be found in the literature about the frequencies of the blood groups in psychiatric disorders we have grouped 611 persons treated for alcoholism.

Alcoholism is a chronic behavior disorder manifested by repeated drinking of alcoholic beverages in excess of the dietary and social uses of the community and to such an extent that it interferes with the drinkers health or his social or economic functioning. Authorities make a distinction between two categories of alcoholics—the alcohol addict or addicted drinker, and the habitual symptomatic excessive drinker. In both groups the excessive drinking is seen as a representation of underlying psychological or social pathology.

The ABO blood groups of 631 alcoholics treated in The Blue Cross Clinic during 1960 and 1961 have been tested. All of them were men and all fitted the above mentioned definition of alcoholism. Their average age when admitted to the clinic was 41.6 years varying between 19 and 68.

Their ABO blood groups were as follows:

	No	Alcoholics percent	Control material percent
A	321	50.86	49.27
B	49	7.61	8.13
AB	22	3.49	3.94
O	240	38.04	38.66

The average figures for Norway are quoted from the extensive work of Hartmann & Lundevall (2) comprising the ABO blood groups of 33 580 persons.

References: 1 Race R R & Sanger Ruth. Blood Groups in Man. Blackwell Oxford 3 ed 1958—2 Hartmann O & Lundevall J U. Skr Norske Vidensk. Akad 1944 1. Mat. Naturv. Klasse no 2.

